MORPHOMETRY OF MUSCULAR PULMONARY ARTERIES WITH SPECIAL REFERENCE TO THE EFFECTS OF AGE AND SMOKING

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DECLARATION

Except where otherwise indicated I carried out the work described herein. As such I accept full responsibility for any errors in, or omissions from this holograph.

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ABSTRACT

Structural changes in the pulmonary vessels are usually first evident in the muscular pulmonary arteries. Current methods for quantitating the media, intima and artery size are inadequate because they produce measurements that are affected by artery collapse/constriction or the irregular distribution of intimal abnormality within and between vessels, or they are unacceptably tedious. New techniques were developed in this study to overcome these problems; using a semi-automatic digitising system measurements are produced, directly from histological sections, of medial and intimal area, and artery size is defined as total length of internal elastic lamina (IEL).

Technique validation was carried out on thirteen subjects with a variety of cardio-pulmonary disorders. At an appropriate magnification reproducibility of the measurements is excellent. Although only cross-sectional arteries with a well-defined IEL ('digitisable') may be measured these are representative of the total population.

The relationship between medial area and artery size is a curved one, best linearised by taking the square root of medial area. This latter relationship was used to investigate the effect of different tissue preparation procedures on the measurements obtained, specifically by comparing slopes of the regression lines. Complete arterial distension by an injection medium caused the IEL to stretch, x 1.5. However, neither the pressure/method used for

lung inflation/fixation nor the tissue embedding medium affected the above relationship. Tissue shrinkage was considerable with paraffin embedding/sectioning and negligible with glycol methacrylate in which more arteries were considered 'digitisable'.

A new method for expressing intimal abnormality was devised, the Intima Index, namely intimal area expressed as a proportion of the area enclosed by the IEL in its theoretically unwrinkled state. Values range from >0 to $\leqslant 1$ indicating minimal through to total lumen occlusion. Subjects are compared by calculating mean Intima Indices for arteries sub-divided by size.

The medial and intimal areas of cross-sectional but 'undigitisable' arteries may be obtained by simply delineating the boundaries of intima-media, and media-adventitia. The total length of the IEL in such arteries is readily obtained by multiplying the length of the intima-media boundary by a factor based on a by-eye estimate of the degree of lamina 'crinkling'.

The effects of age and smoking on muscular pulmonary arteries were studied in thirty-two resection specimens and twenty-three autopsy specimens. The amount of medial muscle was unaffected by age, sex or smoking habit, and varied considerably between subjects. In small arteries it correlated with absolute weight of right ventricle.

In the autopsy group the amount of intimal abnormality, although very varied, increased with age in all sizes of artery but was most prominent in the smaller ones; contrary to previous

reports no lobar differences were evident. These trends were strongest in smokers.

The resection group was unsuitable for this study, the results suggesting that superimposed on the effects of age and smoking there is a degree of intimal fibrosis which results either directly or indirectly from the presence of the bronchial carcinoma itself.

ABBREVIATIONS, UNITS AND SYMBOLS

CO_2	carbon dioxide
D	diameter
EEL	external elastic lamina
GMA	glycol methacrylate
H_2O	water
IA	intimal area
IEL	internal elastic lamina
II	Intima Index
LA	lumen area
LC	lumen circumference
MA	medial area
MT	medial thickness
n	number
O_2	oxygen
OP	over proof
p	probability value
RV	right ventricle
^oC	degrees Centrigrade
cm	centimetre
cm H_2O	centimetres water pressure
g	gram
K	kilobyte
mm	millimetre
mm Hg	millimetres mercury pressure
ml	millilitre
oz	ounce
sq cm	square centimetre
µm	micron
"	inch
%	percentage
<	less than
>	greater than
⩽	less than or equal to
⩾	greater than or equal to
π	pi

PREFACE

This thesis comprises three chapters. It is primarily concerned with methods of measuring the structural components of pulmonary blood vessels (Chapter 2) and the application of these methods to answering specific questions about the effects of age and smoking on the pulmonary vasculature (Chapter 3). When debating what should be included in the thesis it was felt that readers might appreciate a brief review of the normal anatomy and histological structure of the various component vessels of the pulmonary circulation before venturing into the two areas described above. This forms the basis of the introductory Chapter 1.

At the beginning of each of the three chapters the reader will find a short description of the lay-out of that chapter.

CHAPTER 1

THE HUMAN PULMONARY CIRCULATION

The purpose of this chapter is to provide a description of the normal anatomy and histological structure of the various blood vessels which comprise the pulmonary circulation. It starts with a brief account of the important discoveries about the circulation of the lungs and a description of that circulation. These two sections are followed by an account of the anatomy and histological structure of its component blood vessels. Finally there are sections on how the function of the pulmonary circulation may be altered in certain disease states and why there is such interest in measuring the structural components of its vessels.

1.1 HISTORICAL ASPECTS

As early as the second century A.D. it had been shown by Galen that blood passed from the right side of the heart through the pulmonary arteries to the lungs, returning to the left side of the heart through the pulmonary veins. The interaction of pulmonary blood flow and respiration was not recognised until the 13th century, however, following the research of Ibn Nafis in Cairo. Final acceptance that the pulmonary circulation was subservient to the prime function of the lungs, respiration, came with the observations of Harvey, and Malpighi's discovery of the pulmonary capillaries, both in the 17th century. These two workers established the foundations of our current knowledge of the pulmonary circulation.

1.2 CIRCULATION OF THE LUNGS

In terms of blood supply the lungs are unique, there being two separate circulations, the bronchial circulation (a systemic circulation) and the pulmonary circulation. The bronchial circulation makes only a modest contribution to the overall blood supply of the lungs. Its function is to nourish the broncho-vascular bundles and it provides oxygenated blood by way of the bronchial arteries. Blood supply to the lungs is almost exclusively provided by the pulmonary circulation. This circulation carries the entire output of the right ventricle of the heart making the lungs the most richly supplied body organs in terms of total blood flow received. Unlike the bronchial circulation the pulmonary circulation benefits the body as a whole since its function is to assist in the gaseous exchange of O_2 and CO_2 with the atmosphere.

With the advent of cardiac catheterisation techniques (Cournand et al., 1944) it became possible to measure the pressures existing in the pulmonary circulation. In normal man the resting pulmonary arterial pressure is fairly constant throughout adult life showing a mean value of approximately 15mm Hg with systolic and diastolic pressures of approximately 22mm Hg and 10mm Hg respectively (Harris & Heath, 1962); these pressures are considerably lower than those existing in the systemic circulation. The difference in pressures of the two circulations is reflected in the structure of nearly all their component parts. In general pulmonary vessels are thinner walled and wider than systemic vessels in keeping with the lower pressures and greater flow within them.

1.3 ANATOMY AND HISTOLOGICAL STRUCTURE OF VESSELS IN THE PULMONARY CIRCULATION

1.3.1 Age Differences

Any description of the anatomy and histological structure of the pulmonary blood vessels would be incomplete if there were no mention of age differences, the most striking of which exist between the foetal/perinatal period and the young adult. These differences are a reflection of the different haemodynamic situations existing in the pulmonary circulation at these times. During foetal life the foetus derives its oxygen from the placenta and the lungs have no function with respect to blood oxygenation. The arrangement of vascular channels in the heart at this time is such that only 10-15% of the output of the right ventricle actually goes to the lungs (Rudolph, 1970), compared to 100% after birth when oxygenation of blood becomes localised in the lungs. These two entirely different pulmonary haemodynamic situations, one a low blood flow and a high vascular resistance, the other a high flow and a low resistance, not surprisingly require vessels with different structural characteristics.

There are also characteristic changes associated with ageing (Brenner, 1935a) which may be considered normal rather than pathological although it should be pointed out that they are common only in the Western world. A description of these age changes is deferred until Chapter 3.

The following description of anatomy and histological structure refers to pulmonary blood vessels in the young adult; it was not thought necessary to provide a description of the vessels during the foetal/perinatal period since this is of no direct relevance to the thesis. The vessels have been sub-divided into three main classes starting with the extrapulmonary arteries supplying deoxygenated blood to the lungs from the right ventricle, and finishing with the extrapulmonary veins draining oxygenated blood into the left atrium. In between the two are the intrapulmonary blood vessels. Vessels in this class have been further sub-divided into different anatomical types for each of which there is a description of vessel course and histological structure where pertinent.

1.3.2 Extrapulmonary Arteries

Included in the extrapulmonary arteries are the pulmonary trunk and the main pulmonary arteries. The pulmonary trunk arises from the base of the right ventricle of the heart in front of the aortic orifice but slightly above and to the left. Its orifice is circular and provided with a pulmonary valve. Corresponding to the three cusps of this valve there is a slight dilatation of the initial portion of the trunk, its sinus. From its origin at the base of the right ventricle the pulmonary trunk follows an obliquely upward course for approximately 4.5cm before dividing into the left and right main pulmonary arteries. This bifurcation is located just under the aortic arch.

The left main pulmonary artery is a continuation of the pulmonary trunk. On its path towards the hilum of the left lung it curves over the left upper lobe bronchus at the site of its origin from the left main bronchus.

The right main pulmonary artery arises at right angles from the pulmonary trunk. It follows a horizontal course to the right, behind the ascending aorta, reaching the hilum of the right lung in front of the right main bronchus.

To put the size of these vessels into perspective the lumen diameter of the pulmonary trunk is of the same order as that of the ascending aorta, about 3cm. Its wall thickness is less, however, generally measuring only 60-75% of the aortic wall thickness (Heath et al., 1959).

In terms of histological structure the pulmonary trunk and the main pulmonary arteries are essentially similar and comprise three distinct layers, the intima, media and adventitia.

The intima is a very thin layer consisting of a single endothelial layer overlying a basement membrane.

The media is described as being of the elastic type, i.e. there is a significant amount of elastic tissue present in the form of elastic laminae. In the pulmonary trunk these elastic laminae tend to be interrupted and fragmented in contrast to the intact and parallel arrangement present in the aorta. There is great variation, however, in the elastic configuration of the pulmonary trunk in different individuals, ranging between the two extremes

just described. The spaces between the elastic fibres in the media contain acid mucopolysaccharides and a variable but significant amount of collagen fibres. Also present are smooth muscle cells.

The adventitia is composed of fibrous tissue and contains vasa vasorum supplying the pulmonary trunk. Such a vascular system is necessary because the thickness of the media is greater than the distance across which oxygen can diffuse at a sufficient rate from the lumen. The function of these vasa vasorum, which originate mainly from the left coronary artery (Parke, 1970), is to penetrate from the adventitia to supply oxygen to the smooth muscle cells of the media.

The fibro-elastic structure of the pulmonary trunk and main pulmonary arteries is consistent with their function which is the conduction and containment of blood.

1.3.3 Intrapulmonary Blood Vessels

This group of vessels consists of six different types: elastic arteries, muscular arteries, arterioles, alveolar capillaries, venules and veins. Much of our knowledge of the course and branching structure of these vessels has been gained from post-mortem arteriograms and corrosion casts.

With regard to the pulmonary artery system its structure and function have been reappraised by Reid (1968). Branches of the pulmonary arterial tree follow a rather inconsistent dichotomous system in close association with ramifications of the bronchial tree

and in histological sections the vessels can be seen situated next to the bronchi and bronchioles down to the level where the respiratory bronchioles break up into alveolar sacs. As well as these conventional branches there are the so-called supernumerary branches which arise perpendicularly from main conventional branches (Cumming et al., 1969; Elliott & Reid, 1965); these supernumerary branches do not accompany the bronchial tree. They are fairly numerous, so much so that in the periphery of the lung they often outnumber the conventional branches (Elliott & Reid, 1965).

On the venous side of the pulmonary circulation smaller branches unite to form progressively larger veins which are found in close association with the interlobular septa.

(i) Elastic pulmonary arteries

On entering the hilum of each lung the main pulmonary arteries divide into lobar and then segmental arteries. The number and course of these arteries is so varied that there is no 'normal' pattern (Cory & Valentine, 1959; Wagenvoort & Wagenvoort, 1977). Elastic pulmonary arteries are found down to branches with an external diameter of approximately 1000μm (Brenner, 1935a). The internal elastic lamina and other laminae in the media are concentrically arranged, the number present depending on the size of the artery, e.g. in arteries measuring 5000μm in diameter there are usually 16-20 elastic laminae but only three or four remain by the time branches with an external diameter of 1000μm are reached (Harris & Heath, 1977).

(ii) Muscular pulmonary arteries

From a diameter of 1000μm down to about 500μm there is a transition from elastic to muscular pulmonary arteries. This is the point at which the elastic laminae within the media disappear completely with the exception of the prominent internal and less prominent external elastic laminae which bound the medial (muscle) layer. Muscular pulmonary arteries persist down to branches with an external diameter of approximately 70μm (Brenner, 1935a).

The intima of muscular pulmonary arteries is normally a thin layer consisting of an endothelium resting on a basement membrane and overlying just a few reticulin and collagen fibres.

The media is also a thin layer, thin at least in comparison to comparable systemic vessels. As already mentioned, it is bounded on both sides by an elastic lamina. Smooth muscle cells comprise the bulk of the medial layer; these are circularly or nearly circularly orientated. Between the smooth muscle cells there are scanty reticulin and collagen fibres. In some of the larger muscular pulmonary arteries there are also occasional thin elastic fibres.

The adventitia of the larger muscular pulmonary arteries consists of rather dense fibrous tissue which is often two or three times the thickness of the medial layer. There is a progressive thinning of this layer as the artery branches diminish in size.

The structure of muscular pulmonary arteries, thin-walled with a wide lumen (illustrated in Figure 1.1) is in keeping with the low pressures and low resistance to blood flow, which are character-

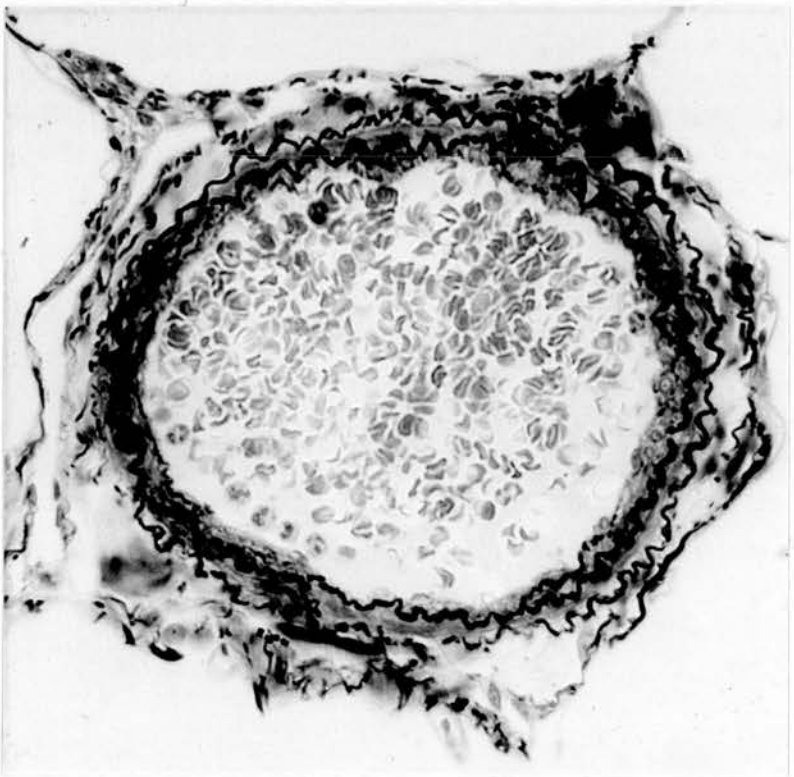


Figure 1.1 A typical muscular pulmonary artery.
x 450
Elastic Stain

The medial component is bounded by a prominent internal and less prominent external elastic lamina. The intima lies internal to the internal elastic lamina.

istics of the pulmonary circulation. It is perhaps pertinent to point out here that although pulmonary blood flow is much greater in the lower than in the upper lobes of the lung (West & Dollery, 1960), there are no reported differences in the structure of pulmonary vessels in different lobes (Simons & Reid, 1969; Wagenvoort & Wagenvoort, 1965a), at least with respect to the media.

(iii) Pulmonary arterioles

Muscular pulmonary arteries start losing their muscular wall at an external diameter in the region of 100μm. By the time branches of 70μm external diameter are reached the muscle layer has usually completely disappeared. This transition is not a sharp one; the circularly orientated smooth muscle cells within the media follow a progressively more widening spiral course resulting in arteries which are 'partially muscular' when cut in cross-section. When the muscle coat is lost completely the vessels are termed arterioles. Unfortunately, there is still some confusion over the usage of the term 'arteriole', which arises mainly from its very different definition in the systemic circulation. Some workers use it to describe a vessel with no muscular coat (Wade & Ball, 1957) but others (Brenner, 1935a) define an arteriole in terms of the external diameter of the vessel (less than 100μm) referring to muscular and non-muscular arterioles as the case may be. Needless to say this creates a great deal of confusion which is compounded by the fact that pulmonary 'arterioles' or 'non-muscular arterioles' are indistinguishable from pulmonary venules. This problem is raised again in the forthcoming section on pulmonary venules (v).

The wall of pulmonary arterioles is simple in structure consisting basically of a single elastic lamina lined on its inner side by an intima. A thin endothelial layer comprises the latter; this layer rests on a basement membrane, overlying scanty collagen and reticulin fibres.

(iv) Alveolar capillaries

Dense networks of alveolar capillaries are the main constituents of the alveolar walls in the lung (Miller, 1947). These networks are supplied by blood from terminal branches of arterioles and are drained by very small pulmonary venules. The meshes within these capillary networks form roughly hexagonal structures, each of the six sides consisting of a capillary segment which is about 10-15μm long and 8.3μm on average in diameter (Weibel & Gomez, 1962; Weibel, 1963). There is some communication between pulmonary capillary networks in adjacent alveoli (Knisely, 1960; Staub, 1963) so that blood perfuses several alveoli before going into the venous system.

The wall of the alveolar capillary, which in effect constitutes the air-blood barrier, consists of five layers but is extremely thin, 1.6-1.8μm, (Weibel, 1970b). On the side nearest the alveolar space the wall is lined by an attenuated cytoplasmic layer of epithelial cells. Bordering the capillary lumen on the other side there is an equally thin cytoplasmic layer formed by endothelial cells (Low, 1961; Wagenvoort & Wagenvoort, 1977). Both these layers rest on thin basement membranes which are sometimes indistinct. Sometimes

the two basement membranes fuse but more often there is a thin interstitial space between them containing some reticulin and collagen fibres (Wagenvoort & Wagenvoort, 1977). The structure of the lung at this level and how it affect gaseous exchange is described in detail by Weibel (1983 a and b).

(v) Pulmonary venules

Blood is drained from the capillary networks by collecting venules which merge to form wider pulmonary venules (Reeves et al., 1965). As mentioned earlier pulmonary venules are indistinguishable from pulmonary arterioles except by serial sectioning.

(vi) Pulmonary veins

The venules merge to form larger veins which are usually found within the interlobular fibrous septa. As successive tributary veins are accepted into the pulmonary venous drainage system the pulmonary veins increase in calibre. Finally, one lobar pulmonary vein drains each lobe. As with the lobar arteries the course and pattern of lobar veins varies so much that there is no 'normal' pattern.

In terms of histological structure pulmonary veins are distinctly different from pulmonary arteries. Veins of different sizes, however, have essentially the same structure.

The intima comprises a thin, endothelial layer resting on a basement membrane.

The medial layer is thinner than that of arteries and consists of irregularly arranged elastic fibres interspersed with smooth muscle cells and collagen (Wagenvoort, 1970). An internal elastic lamina is normally recognisable but there is no external elastic lamina, making the boundary between the media and adventitia indistinct.

The adventitia is a thin layer of collagenous and elastic fibres.

1.3.4 Extrapulmonary Veins

Despite the variations in the course and pattern of the lobar veins it is usual for two large pulmonary venous trunks to emerge at the hilum of each lung. As their names suggest the superior veins drain blood from the upper or upper plus middle lobes; the inferior veins drain the lower lobes. These large venous trunks run a short course before entering the upper part of the left atrium in the heart.

The extrapulmonary part of the venous system is somewhat different from the intrapulmonary part in that the venous trunks are surrounded by a coat of cardiac (striated) muscle which extends from the wall of the left atrium. Normally this coat abruptly disappears at the hilum of each lung (Nathan & Eliakim, 1966).

1.4 THE BRONCHIAL CIRCULATION

Having dismissed the bronchial circulation almost out of hand earlier on in this chapter, it is now appropriate to return to it. Workers engaged in studies of the pulmonary circulation must consider the bronchial circulation for two main reasons. First of all they must be able to identify the bronchial vessels in order to distinguish them from those in the pulmonary circulation, and secondly, anastomoses between vessels in the two circulations do occur (Marchand et al., 1950). These two areas are dealt with briefly in the following two sections.

1.5 ANATOMY AND HISTOLOGICAL STRUCTURE OF VESSELS IN THE BRONCHIAL CIRCULATION

In the extrapulmonary part of the bronchial circulation there is considerable variation in both the number and origin of the bronchial arteries (Miller, 1947; Tobin, 1952). Once in the lung, however, the bronchial arteries accompany the bronchi and their divisions (Cudkowicz & Armstrong, 1951), their function being to supply the bronchial walls with oxygen. When the bronchi lose their cartilage the bronchial arteries disappear, breaking up into capillary networks situated both within and outside the muscle coat of the bronchial wall (Miller, 1947). From the capillaries blood is drained through bronchial venules which unite to form plexuses around the bronchi. It is from these plexuses that the bronchial veins arise. At this point there are wide anastomosing connections between the bronchial and pulmonary veins (Marchand et al., 1950),

and blood from the bronchial veins is drained via the pulmonary veins into the left atrium. These bronchial veins are termed the central bronchial veins (Marchand et al., 1950). In addition to these, there are bronchial veins arising from both the pleura and hilum of the lung, the so-called peripheral bronchial veins or pleuro-hilar veins (Marchand et al., 1950). Blood from these peripheral bronchial veins is drained into the right atrium.

In terms of histological structure, the bronchial arteries show the same features as other arteries in the systemic circulation, i.e. they are much thicker-walled than the pulmonary arteries. The intima is usually a single layer of endothelial cells which is separated from a thick muscular media by a well-defined internal elastic lamina. The external elastic lamina, if present at all, is thin and fragmented.

Distinguishing bronchial veins from the pulmonary veins is much more difficult since they are essentially the same, although the bronchial veins tend to be that bit thinner-walled.

1.6 BRONCHO-PULMONARY ANASTOMOSES

In the normal lung the most common vascular anastomosis is between the bronchial and pulmonary veins as described in section 1.5, and although broncho-pulmonary arterial anastomoses do sometimes occur, their functional significance is considered negligible. There is still some confusion as to whether there are any arterio-venous anastomoses in the normal or non-diseased lung but most workers seem to think not.

Under certain pathological conditions, especially those in which blood flow through the pulmonary arteries is restricted or obstructed, there may be an increase in the number of arterial broncho-pulmonary anastomoses (Liebow et al., 1948; Liebow et al., 1949). These have important functional implications since they are likely to affect the haemodynamics of the pulmonary circulation by increasing the pressure within it and contributing to pulmonary hypertension.

1.7 FUNCTIONAL ASPECTS OF THE PULMONARY CIRCULATION

This section has deliberately been kept brief, only general principles being considered, because the subject matter is really outwith the scope of the thesis. Nevertheless it serves as a very useful introduction to the forthcoming chapters. Much of the information presented in this section was obtained from three excellent textbooks: The Human Pulmonary Circulation (Harris & Heath, 1977), Pulmonary Vascular Diseases (Moser (Ed.), 1979) and Pathology of Pulmonary Hypertension (Wagenvoort & Wagenvoort, 1977).

As a starting point to this section two questions may be asked - "What factors determine the pulmonary arterial pressure?" and "What are the causes and effects of an increased pulmonary arterial pressure?"

Briefly, two factors determine the pulmonary arterial pressure; these are blood flow and vascular resistance to that flow. When the pulmonary arterial pressure is raised beyond its normal limits such that its mean value exceeds 25mm Hg, or its systolic-diastolic

pressures exceed 30/15mm Hg, then the condition pulmonary hypertension is said to be present. If this is sustained it can lead to right ventricular hypertrophy and possibly even right heart failure. Knowing this it is not then difficult to understand the importance of maintaining a normal pulmonary arterial pressure and why there is such interest in determining how pulmonary hypertension arises.

In theory, pulmonary hypertension could result from an increased blood flow; alternatively it could result from an increased vascular resistance to blood flow. In physiological terms the latter could be due to reduction in size of the pulmonary capillary bed, functional alterations involving the calibre of the pulmonary vessels, or obstruction to pulmonary vessels, either internal or external (Lamb, 1975). Because the pulmonary circulation is a low pressure - low resistance system, an increase in cardiac output (as with exercise) does not produce an increase in the pulmonary artery pressure until the output has increased threefold. Once this value is exceeded there is a relatively steep linear rise in pressure in proportion to blood flow. The ability of the pulmonary circulation to accept an increased blood flow without increased pressure stems from the highly distensible nature of the pulmonary vessels, which is a feature of their structure, and which is enhanced by their relatively thin walls. It also has important implications. More than 50% of the pulmonary vascular bed must be destroyed, removed (pulmonary resection) or obstructed before pulmonary hypertension is present at rest. Damage less severe than this will reduce the reserve so that the pulmonary artery pressure

will rise with less of an increase in cardiac output. Once the pulmonary artery pressure is raised at rest, any increase in blood flow will produce a further increase in pulmonary artery pressure.

Although severe damage to the lung is required to destroy more than 50% of the pulmonary vascular bed, an increased resistance in the pre-capillary vessels (muscular pulmonary arteries and arterioles) will readily cause a rise in the pulmonary artery pressure. This increased resistance, brought about by a decrease in vessel calibre, may be functional or organic, that is to say the vessels may actively constrict or they may be physically narrowed by, for example, occlusive thromboemboli or intimal fibrosis. When one considers that resistance is inversely proportional to the fourth power of the vessel radius (Poiseuille's Law) then the importance of even very minor changes in vessel calibre is realised.

With regard to active vasoconstriction two questions spring naturally to mind - "What is the stimulus?" and "What is the effect?" In principle the stimulus may be nervous, chemical or humoral. Nervous regulation is rather unlikely, however, since nerve fibres are difficult to demonstrate in the muscular pulmonary arteries (Mitchell, 1956 as quoted by Wagenvoort & Wagenvoort, 1977), and although evidence for a humoral regulation of the pulmonary circulation is as yet incomplete, the evidence there is suggests that humoral factors do not play an important role.

As the chief function of the pulmonary circulation is to assist in the gaseous exchange of CO_2 and O_2 with the atmosphere, it seems logical that a low alveolar O_2 tension (or a high CO_2 tension) can

cause local pulmonary vasoconstriction leading to a rise in pulmonary arterial pressure. Hypoxia is the most potent vasoconstrictive agent known and it is thought that it acts, not through an intermediary, but directly on the vascular smooth muscle cells. Chronic hypoxia is one of the commonest causes of pulmonary hypertension and is found in patients with chronic obstructive lung disease, and also in individuals with no pre-existing structural abnormalities of vessels, living at high altitudes where there are low barometric pressures.

Sustained vasoconstriction may bring about structural changes in the pulmonary arteries, the earliest of which is usually medial (muscle) hypertrophy, or more correctly hyperplasia. Medial hypertrophy may manifest itself as an increase in muscle in existing muscular arteries and/or as an extension of muscle into previously non-muscular vessels. Whatever the mechanism, the increased amount of muscle endows the muscular arteries with even greater constrictive powers and a vicious circle of increased pulmonary vascular resistance and increased pressure may be entered into. Although intimal changes generally take more time they may become very prominent, further reducing vessel calibre with severe and lasting consequences for the pulmonary circulation.

Generally speaking, pulmonary hypertension can be linked to a specific disease or abnormality of either the heart or lungs, although not always, as is the case in primary pulmonary hypertension. To list all the possible diseases or abnormalities would be too time-consuming but fortunately they can be grouped, each

group characterised by a common aetiological factor. A list of the factors is given in Table 1.1.

While it is true that the underlying disease or abnormality is usually known, it is perhaps the only area of certainty with regard to the subject of pulmonary hypertension. Uncertainty surrounds two particularly important areas. The first of these concerns the mechanism(s) by which pulmonary hypertension is brought about. This area has been and continues to be the subject of extensive research. The second area of uncertainty concerns the effect of pulmonary hypertension on the pulmonary vessels, in particular those vessels in which the major site of pulmonary vascular resistance is found, the muscular pulmonary arteries and arterioles. This topic is further pursued in the following section.

1.8 THE IMPORTANCE OF QUANTITATIVE STUDIES OF THE PULMONARY VASCULATURE

In all cases of pulmonary hypertension workers wish to establish whether or not the functional aberrations in the pulmonary circulation are related to structural changes in the pulmonary vessels, and if there are structural changes, to what extent they have progressed. Such an evaluation may be required to be done on post-mortem material or on biopsy specimens. All that is needed is a way of assessing both the medial and intimal components of the pulmonary vessels; in practice this is less than easy. A number of workers have approached the problem by devising grading systems (Heath & Edwards, 1958; Wagenvoort, 1973; Wagenvoort, 1974; Yamaki & Tezuka, 1976) for assessment of the media and intima. Without going

Table 1.1 Causes of pulmonary arterial hypertension.

Pre-tricuspid shunts
Post-tricuspid shunts
Common atrioventricular canal and pre- and post-tricuspid shunts
Prolonged left atrial hypertension
Chronic hypoxia
Pulmonary fibrosis
Recurrent pulmonary embolism/thrombosis
Primary pulmonary hypertension
Diet, e.g. pyrrolizidine alkaloids
Hepatic disease
Filariasis
Lung worm
Pulmonary arterial stenosis

into these systems in detail, it can be said that, depending on the particular system, workers are required to make subjective decisions on, amongst other factors, the presence/extent of medial hypertrophy, the type of intimal fibrosis and degree of lumen occlusion. This sort of approach is not a particularly sound one. Casting aside the subjectivity aspect, the real problem is that assessment of medial hypertrophy or lumen occlusion is very much influenced by the degree of constriction, or for that matter post-mortem collapse of the artery. Very constricted/collapsed arteries appear to have very thick walls and narrow lumina; in such situations even the media of normal vessels would be considered to be hypertrophied. In view of this, one must cast some doubt on whether medial hypertrophy is truly as common in cases of pulmonary hypertension, especially those involving chronic hypoxia, as one is led to believe. Confirmation is possible only through the application of precise measuring techniques and a knowledge of the range of values observed in the 'normal' state, the latter acquired by the same techniques. Determination of the 'normal' range requires an assessment of the effects of age and smoking on the pulmonary vessels, and an assessment of whether there are any sex differences.

Particularly over the last 25 years or so there have been many quantitative studies of the medial, and to a lesser extent, intimal component of pulmonary vessels, especially the muscular pulmonary arteries, in a variety of disease states (e.g. Hale et al., 1980; Hasleton et al., 1968; Haworth & Reid, 1977a; Heath & Best, 1958; Naeye, 1966; Wagenvoort, 1960; Wagenvoort & Wagenvoort, 1965b). In

the course of these studies a variety of measuring techniques have been applied; few of these have been precise, that is to say few have yielded measurements of media, intima and especially artery size, that were unaffected by the degree of constriction/collapse present. Because of this the results of many of these 'quantitative' studies are perhaps just as suspect as those obtained using simple grading systems.

From the efforts of workers over the last 25 years it is obvious that there is still enormous scope for the development of new methods for measuring the medial and intimal components of pulmonary arteries and their size, methods which produce accurate measurements. Without the development of these techniques, it remains difficult to assess the true extent of structural alterations in the pulmonary vessels in disease.

The technical aspects of the techniques used for quantitating the media and intima are reviewed in the following chapter.

CHAPTER 2

TISSUE PREPARATION TECHNIQUES AND MEASURING TECHNIQUES USED IN STUDIES OF THE PULMONARY VASCULATURE

Chapter 2 comprises five main sections: an Introduction, the Aims of the chapter, a Material and Methods, Results and Discussion. The two main themes of this chapter are the methods used in the preparation of lung tissue for study of the pulmonary vasculature, and the techniques used for assessing the medial and intimal components of the pulmonary arteries.

2.1 INTRODUCTION

The purpose of the Introduction is to detail first of all the various methods that are used to prepare lung tissue for study of the pulmonary arteries, isolating those factors with the potential to affect, by whatever means, the measurements obtained. The factors to be considered are:-

1. Arterial distension with injection media
2. Tissue fixation
3. Sampling
4. Tissue embedding
5. Tissue sectioning
6. Tissue staining

Following on from this, the techniques that have been reported for measuring the medial and intimal components of pulmonary arteries will be reviewed and some comment made on their usefulness. It should be pointed out that in this particular section mention will be made of studies involving the systemic circulation where relevant.

2.1.1 Methods in Tissue Preparation

When preparing lung tissue for the histological examination of pulmonary arteries there are several standard steps that must be taken and of prime importance is tissue fixation. Following on from this it is necessary to sample the lung, selecting a number of tissue blocks which must be embedded, sectioned and stained. Some

workers in the field of pulmonary or systemic arteries include an additional optional step, that of distension of the arteries with an injection medium.

(i) Arterial distension

The purpose of this procedure is usually twofold; by using a radiopaque injection medium it is possible to x-ray the lung and obtain a permanent record of the course and distribution of the pulmonary arteries so that patterns in disease states may be studied. Most workers who distend the pulmonary arteries, however, do so in an attempt to overcome the problems of variable post-mortem collapse or constriction of arteries, which make diameter a rather imprecise indicator of size. (This problem is expanded upon further in section 2.1.2 (ii)). An added bonus of arterial distension with an injection medium is that it is easy to distinguish the pulmonary arteries from both the bronchial arteries and the pulmonary veins since neither of these two classes of vessel is penetrated by the injection medium.

Various injection media have been used including a gelatin solution (Naeye, 1965a), a gelatin and India ink mixture (Lodi & Viswanathan, 1974) and a bismuth oxychloride gelatin suspension (Short, 1956; Short, 1957; Short & Thomson, 1959). Some workers have even tried using radioactive injection mixtures (James et al., 1960). The most commonly used injection media, however, contain a mixture of gelatin and barium sulphate as their main ingredients. This type of radiopaque mixture was first used in 1924 by Hinman & Morison in a study involving the systemic circulation and since then

it has been used in several other such studies, e.g. Cook & Yates (1972). Short has used it in studies of the pulmonary circulation in 1956 and 1957 but its use in this field is characteristic of one research group in particular, that headed by Lynne Reid. She and her fellow workers have carried out arterial distension as a routine part of tissue preparation in countless studies of the human pulmonary circulation, both adult (e.g. Anderson et al., 1973; Hislop & Reid, 1973a; Ryland & Reid, 1975; Shelton et al., 1977) and foetal/new born (e.g. Haworth & Reid, 1977a; Haworth & Reid, 1977b; Hislop et al., 1975), and also in their studies using the rat as a model of the human pulmonary circulation (e.g. Hislop & Reid, 1974; Hislop & Reid, 1978; Meyrick & Reid, 1981; Meyrick & Reid, 1982).

There are problems associated with the use of gelatin based injection mixtures in that they must be kept at fairly high temperatures or they rapidly solidify. Reid and her associates have carried out arterial distension with the injection medium at a temperature of 60°C (Reid, 1967); other workers have used even higher temperatures, e.g. 80°C (Short, 1956; Short, 1957). Such high temperatures necessitate warming the specimens beforehand to avoid early solidification of the injection medium; this is usually carried out by bringing the specimens to room temperature and then heating them to 37°C (Reid, 1967) or 40°C (Short, 1956; Short, 1957) in a water bath. These problems can be avoided by using 'cold' gelatin mixtures such as Chromopaque (Stigol et al., 1969) or that described by Schlesinger (1957). This mixture remains liquid at room temperature because its gel point is depressed by the addition of potassium iodide; by predetermining the amount of formalin added

to the mixture it is possible to vary its solidification time. Although Carrington (1968) has advocated the use of 'cold' rather than 'hot' gelatin mixtures in his review of injection methods in the study of pulmonary disease, there has been a surprising reluctance to use them in studies of the pulmonary circulation.

The biggest problem encountered by workers using an injection medium for distension of pulmonary arteries is deciding what distension pressure should be used. Basically there are two options, a standard pressure can be used, or the injection can be carried out at the pressure recorded during life using cardiac catheterisation techniques. Although some studies using the latter have been reported, e.g. Reeves et al. (1966) and Wagenvoort (1960), this information is so rarely available that the vast majority of workers have had no option but to use a standard pressure. Some have used different standard pressures depending on the presence or absence of pulmonary hypertension, e.g. 100mm Hg and 50mm Hg respectively (Short, 1956; Short, 1957). The most common practice, however, and that adopted by Reid, is to use a set hypertensive pressure for all specimens, generally 100cm of H_2O (Reid, 1967; Warnock & Kunzmann, 1977a), which causes complete distension of all arteries. Reid and colleagues use this pressure for both humans and rats regardless of the cardio-pulmonary pathology present.

In conclusion, the following comments may be made with regard to arterial distension with injection media. It is likely that this procedure does have a significant effect on the pulmonary arteries; not only are unnatural substances being injected into them but they

are being injected generally at very high temperatures and at pressures that do not normally exist in the pulmonary circulation.

(ii) Lung inflation/fixation

This is usually the first step in the tissue preparation process unless arterial distension is carried out. 10% solutions of formaldehyde in either water (formalin) or normal saline (formol saline) head the list of the most widely used fixatives; these solutions are sometimes buffered. Other fixatives that have been used are Zenker's solution (Naeye, 1961b; O'Neal et al., 1955), and if electron microscopy is to be carried out, glutaraldehyde (Rabinovitch et al., 1978) or glutaraldehyde in cacodylate buffer (Meyrick & Reid, 1981; Meyrick & Reid, 1982) is usually the fixative of choice.

Although some workers have simply fixed lung tissue by immersion in either formalin (e.g. Heath et al., 1968; Naeye, 1965b; Symchych, 1971; Wagenvoort & Wagenvoort, 1982a) or formol saline (e.g. Enticknap, 1953; Hislop & Reid, 1972; Hunter et al., 1974) it is generally accepted that better specimens are obtained if the lungs are inflated with the fixative. There is no 'best' method for lung inflation/fixation as the best method in any particular situation depends largely on the purpose for which the specimen is to be used. Nevertheless, workers do disagree about what methods, and also what inflation pressures, should be used. Several methods of preparing inflated lung specimens are available, which have been described in detail by Dailey (1973) and Silverton (1965); pertinent comments on the pros and cons of each method are also

given. With regard to studies of the pulmonary vascular bed most workers have opted for the simplest, least expensive and time consuming method, which is that of inflation through the trachea or main bronchi with formalin or formol saline. Normally the inflation is not carried out at any particular pressure but some indication of the extent of inflation is usually given by comments such as "until the pleural surfaces become tense/smooth" (e.g. Abraham et al., 1971; Davies & Reid, 1970; Kay & Heath, 1966) or "until the lungs are fully distended" (James & Thomas, 1968). A number of workers, however, have chosen to carry out this type of inflation at a specific pressure and the range of pressures used reflects the disagreement over which is the best pressure to use. Expressed in cm of H_2O pressures of 20 (Leach et al., 1977), 23 (Meyrick & Reid, 1981; Meyrick & Reid, 1982), 25 (Hale et al., 1980), 36 (Rabinovitch et al., 1979) and up to 45 cm (Haworth & Reid, 1977a; Haworth & Reid, 1977b; Ryland & Reid, 1975) have been reported. In one particular study of the pulmonary arteries of mice (James & Thomas, 1968) the inflation pressure used was 100 cm H_2O; however, pressures as high as this are exceptional.

The time span of these 'constant pressure' inflation techniques is as variable as the pressures used. Pressures have been maintained for five minutes (Leach et al., 1977) or a minimum of 24 hours (Hale et al., 1980), the latter using the constant pressure inflation apparatus described by Heard et al.(1967). With Heard's technique, the pressure is maintained not only throughout the inflation period but also throughout the fixation period. Again this is a matter of some debate but the vast majority of workers are

of the opinion that maintenance of constant pressure throughout the fixation period is unnecessary. The general practice is to allow the lungs to fix by submerging them in fixative for several days (e.g. Arias-Stella & Saldaña, 1962) or more commonly for one week (e.g. Meyrick & Reid, 1981; Semmens, 1970; Semmens & Reid, 1974) following inflation.

Few workers in the field of pulmonary arteries use methods for lung inflation/fixation other than those described, with one notable exception, the research group led by Donald Heath. Although not a standard practice it is fairly common (e.g. Hasleton et al., 1968; Heath et al., 1968; Hicken et al., 1965) for them to prepare inflated lung specimens using the formalin steam method developed by Weibel & Vidone (1961). This technique has the theoretical advantage over those using liquid fixatives in that shrinkage of the alveolar septa does not have to take place over an incompressible fluid core so there is less distortion and flattening of hollow structures such as blood vessels. Despite this advantage it is unlikely that the formalin steam method for lung inflation will ever take precedence over those involving the instillation of liquid fixatives since the latter are so simple and quick to use and require no expensive equipment. It also should be pointed out that the formalin steam method produces excessive tissue shrinkage artefacts (see next page).

It is worthwhile noting that, as a rule, few workers inflate lungs via the main pulmonary arteries, and certainly not when the pulmonary vasculature is to be studied.

As with all other structures in the lung, the most striking effect of lung inflation/fixation on the pulmonary arteries is that of shrinkage and the often concomitant alteration of configuration. Regardless of the method of lung inflation/fixation shrinkage does occur, but to a lesser extent than occurs during the embedding/sectioning process (see section 2.1.1 (iv)). Weibel (1963), for example, quotes correction factors of 1.22, 1.50 and 1.82 for the artificial linear, area and volume shrinkage of lung tissue during fixation using the formalin steam method. These shrinkage factors are unusually high and higher than one would expect using instillation of liquid fixatives. Heard (1958) and Heard et al. (1967) have claimed that with aqueous formalin no evidence of shrinkage could be demonstrated in measurements of lung volumes. This, however, is a fallacy brought about by the fact that in fluid filled lungs some 90% of the lung volume is represented by the instilled fluid and only 10% by the tissue itself. Tissue shrinkage is consequently difficult to detect. As Weibel (1968) points out, there is no doubt that formalin-fixed tissue does shrink. In his view the method of inflation/fixation is of secondary importance as long as all possible care is taken to estimate the magnitude of errors introduced by the method used, and to correct the data accordingly. This is one area in which most studies of the pulmonary vasculature fail; rarely is there any attempt to correct the measurements for shrinkage. Furthermore, there have been no attempts to assess the effect of different inflation/fixation techniques on the measurements obtained.

(iii) Sampling

Sampling of the lung is of immense importance in quantitative studies of the pulmonary vasculature, especially since the lung is a unique organ in two respects. Firstly, the effects of gravity mean that there are differences in blood flow between upper and lower lobes; hence there is potential for differences in the structure of vessels from different lobes. Secondly, of the many diseases known to affect the lung, few affect it to a uniform extent; consequently there is the probability of patchy vascular abnormality. It is, however, impossible to investigate all pulmonary arteries within a lung so samples of tissue have to be taken. In descriptive morphology the usual practice is to sample 'typical' regions which are then subjected to careful investigation. Such a procedure can, however, lead to an overestimation of the importance of some features and sometimes a misinterpretation of their significance. This is also and especially true with regard to quantitative studies of the pulmonary vasculature, in which it is essential to sample the lung in such a way that the arteries within the samples are truly representative of all those within the lung. In those studies using the rat as a model of the human pulmonary circulation (e.g. Heath et al., 1973; Hislop & Reid, 1977; Kay et al., 1967; Meyrick & Reid, 1979), sampling is obviously much less of a problem due to the smallness of the rat lung.

Normally lungs fixed by inflation are sliced into 1cm slices, or 0.5cm in the case of newborn infants or children (Davies & Reid, 1970; Hislop et al., 1975) before being sampled. In practice, there are two fundamental methods of sampling, random sampling (including

stratified random sampling) and systematic sampling, the advantages and disadvantages of which are discussed by Dunnill (1962) and Weibel (1963). The stratified random sampling technique has been validated by Dunnill (1964), and it is generally accepted that random sampling is the more efficient and reliable of the two procedures. It is difficult to determine how sampling is normally carried out in studies of the pulmonary vasculature since in the earlier studies the authors frequently neglected to mention how their tissue blocks had been selected, and for that matter their number and size. Although much less common nowadays, this practice still continues (Matsubara et al., 1984). Where specifically mentioned, sampling is usually random(e.g. Naeye & Laqueur, 1970; Symchych, 1971; Yamaki & Wagenvoort, 1981) or stratified random (e.g. Haworth & Reid, 1977a; Hislop et al., 1975; Ryland & Reid, 1975). Only a few workers have chosen to sample in what might be regarded as a systematic way by taking samples from pre-selected sites within the lung, e.g. Arias-Stella & Saldaña (1962), Hislop & Reid (1972) and Wagenvoort & Wagenvoort (1965a).

Of all the stages involved in the tissue preparation process sampling would appear to show the most variation amongst different workers in spite of the relative consistency in the sampling method used. Some workers have ensured that the tissue blocks are selected from each slice (Hasleton et al., 1968), from each lobe (Berend et al., 1979; Larrabee et al., 1949; Naeye & Dellinger, 1971), maintaining a balance between lobes (Naeye, 1961a), or less often from the various segments of each lung (Wagenvoort, 1960). Since other workers have not imposed these sort of selection criteria

there is consequently a great deal of variation in the number of tissue blocks that are actually taken and examined. Some studies have been carried out on as few as two tissue blocks per case (Ferencz, 1960; Henry, 1952) or as many as 30 (Yamaki & Wagenvoort, 1981). The general policy, however, seems to be to take somewhere between four and six blocks per lung, e.g. Anderson et al. (1973), and Yamaki & Tezuka (1976).

As already stated, it is especially important to ensure that the lung is sampled correctly otherwise there is the danger that the pulmonary artery population selected for study is in some way a biased sample of the total population. In such a situation the measurements obtained might well be suspect.

(iv) Tissue embedding

This is one area, in fact the only area, in which all workers engaged in studies of the pulmonary vasculature follow exactly the same procedure. Without exception all have embedded their tissue samples in paraffin wax.

Alternatives to paraffin are available for tissue embedding, namely the plastic resins such as glycol methacrylate. Their major disadvantage is their cost in technical time, a probable explanation of why they have never been used in any quantitative studies of the pulmonary vasculature, which normally involve a considerable number of subjects and, therefore, tissue blocks. On the plus side, however, there is the much improved quality of tissue sections produced and the concomitant improvement in histological detail,

factors which have long been appreciated (Rosenberg et al., 1960; Wichterle et al., 1960). But undoubtedly the biggest advantage of the plastic resins lies in the fact that they do not cause tissue shrinkage (McLean & Lamb, 1983), a factor which with paraffin embedding is considerable. Depending on the method of tissue fixation, the shrinkage of tissue during paraffin embedding and sectioning is usually greater than that occurring during the fixation process (Tsunoda & Martin, 1973). According to Weibel (1963) and Dunnill (1962) such shrinkage should be taken into account and the appropriate correction factors applied to the measurements obtained. In practice this rarely happens in any quantitative studies and those involving the pulmonary vasculature are no exception.

The considerable tissue shrinkage that occurs with paraffin embedding is of interest for other reasons. It leads to speculation about the shrinkage of the individual tissue components of the pulmonary vessels and the supposition that different tissue components may shrink by differing amounts. If this were the case then it might well affect the measurements of pulmonary arteries obtained in quantitative studies. It is certainly an area worthy of investigation.

(v) Tissue sectioning

Tissue sections are normally cut at a thickness which gives good lateral resolution. In studies of the pulmonary vasculature the most common practice is to take 5μm sections, in the rat as well

as in the human. Some workers take thinner, e.g. 3μm sections (Niwa, 1971; Yamaki & Horiuchi, 1979), whereas others take thicker, up to 7μm sections, in the rat (Wagenvoort et al., 1974; Yamaki et al., 1980) and in the human (Wagenvoort, 1980a; Wagenvoort & Wagenvoort, 1965a; Wagenvoort & Wagenvoort, 1982b). It is unlikely that such differences in section thickness would seriously affect any measurements of pulmonary arteries unless obliquely sectioned arteries were to be included in which case there might well be problems with the thicker sections mainly with regard to possible overlap of the internal and external elastic laminae and trying to distinguish between them.

(vi) Tissue staining

Again this is an area of the tissue preparation process which is unlikely to affect measurements of the pulmonary arteries. For such studies the routine staining of tissue sections with haematoxylin and eosin is of very limited value since it does not stain up the various components of the vascular wall. The most valuable technique is to stain the elastic tissue. Weigart's or Verhoeff's elastic stains are the most commonly used, in conjunction with a van Gieson counterstain. Other widely used elastic stains are Lawson's modification of the Weigart-Sheridan elastic stain or Miller's elastic stain. Some studies have used less well-known elastic stains such as Hart's method for elastic tissue (Henry, 1952) or Gomori's aldehyde fuchsin stain, unmodified (Hunter et al., 1974) or as modified by Humberstone (Leach et al., 1977). However,

the end result of all these staining methods is similar, the elastic tissue, particularly the elastic laminae, are clearly defined.

Some studies, e.g. Wagenvoort & Wagenvoort (1982a), have necessitated staining of the nuclei of the smooth muscle cells in addition to staining of the elastic laminae. If required, this can be achieved by using a combination of Lawson's elastic stain and haematoxylin.

(vii) Summary

Of the six possible steps in the preparation of tissue for quantitative study of the pulmonary arteries, four have the potential to affect the arteries and hence the measurements obtained. These are: arterial distension with injection media, lung inflation/fixation, sampling and tissue embedding/sectioning. Sampling exerts its effect by determining the population of arteries selected for study whereas the remaining three factors have a direct effect on the arteries themselves.

Thickness of tissue section and the staining procedure are considered unlikely to significantly affect measurements of pulmonary arteries.

2.1.2 Methods for Assessing the Medial Component of Pulmonary Arteries

In this section and in section 2.1.3, which concerns the intimal component of pulmonary arteries, the following procedure will be adopted. Initially, there will be a brief description of

'general' methods for 'quantitating' each structural component followed by an account of the more specific methods starting with the simplest techniques and working through to the most sophisticated. At this point there will be no attempt to link the measuring techniques to the methods used in tissue preparation. The reasons for this are quite simple; so many measuring techniques have been used, each of which has been applied to material prepared in such a variety of ways, that it would be impossible to cover them all. The sole objective of sections 2.1.2 and 2.1.3 is to describe in detail the measuring techniques that have been applied in studies of the media and intima respectively of pulmonary (or for that matter systemic) arteries.

(i) The 'general' methods

In any study of the media of pulmonary arteries the main objective is usually to determine whether or not medial hypertrophy has occurred; this may manifest itself as an increase of muscle in existing muscular arteries or as an extension of muscle into previously non-muscularised vessels. It is largely with the latter of these two mechanisms that the 'general' techniques are concerned.

Determination of how far distally muscular arterial tissue extends has been attempted using a number of techniques, the simplest of which involves counting the number and proportion of thick walled peripheral vessels adjacent to alveoli or alveolar ducts (Hunter et al., 1974; Leach et al., 1977; Scott, 1976). The remaining techniques rely on distinguishing different types of

artery and relating them to the type of accompanying airway. This approach has been used by Arias-Stella & Saldaña (1962) but it is most commonly associated with the research groups headed by Lynne Reid, for whom it is an almost standard approach to the assessment of medial hypertrophy both in the human (e.g. Hislop & Reid, 1972; Hislop et al., 1975; Rabinovitch et al., 1980) and in the rat (e.g. Meyrick & Reid, 1982; Meyrick et al., 1980; Rabinovitch et al., 1979). In addition to noting the type of accompanying airway, measurements of the diameter of each artery are made and their structure, e.g. muscular, partially muscular or non-muscular ascertained. From this, information may be obtained on the size of the largest non-muscular artery, smallest muscular artery and the size range of those that are partially muscular. Comments may then be made on whether extension of arterial muscle has occurred.

Grading of the thickness of the muscular walls of pulmonary arteries is another of the 'general' techniques quite commonly used for assessing medial hypertrophy or atrophy, particularly in biopsy specimens. A number of standard grading techniques have been developed which include assessment of the media, such as the Heath-Edwards classification (1958) or those of Wagenvoort (1973) and Yamaki & Tezuka (1976). Other more subjective grading systems have also been applied (e.g. James & Thomas, 1963; Wagenvoort, 1980a; Wagenvoort et al., 1967a); in these medial hypertrophy or atrophy is graded as absent, slight, moderate or severe (James & Thomas, 1963) or as - (normal) through to +++ (severe) (Wagenvoort, 1980a; Wagenvoort et al., 1967a).

The techniques that are of especial interest, as far as this thesis is concerned, are those in which the medial component of the pulmonary (or systemic) arteries has been measured. As Barrett (1963) has pointed out, there are several alternatives open to the researcher who is measuring the media of arteries; thickness or area may be measured and the data presented as they stand or expressed in relation to some parameter which represents artery size.

(ii) The 'wall thickness' methods

Of all the methods for assessing medial hypertrophy, the 'wall thickness' methods are undoubtedly the most frequently used. As their name suggests they involve measurement of the thickness of the medial layer. This is generally, but not always, expressed in relation to some indicator of artery size for which external diameter or lumen diameter are the parameters normally chosen. These measurements are done either using a calibrated graticule in the focussing eye-piece of the microscope or less commonly from tracings of the arteries.

In theory the 'wall thickness' methods are very straightforward since all that is required is a measurement of medial thickness and artery size. In practice they are somewhat less than straightforward since there are differences of opinion amongst workers with respect to:-

1. how to measure medial thickness
2. how to define artery size
3. which arteries to measure

4. how to express the data

To assist in the description of these differences a diagrammatic representation of a muscular pulmonary artery in the uninjected or injected state has been produced (Figure 2.1 (a) and (b) respectively).

It is appropriate to begin this account of the 'wall thickness' methods by referring to those studies in which the pulmonary arteries have not been distended by an injection medium since this is the least sophisticated approach. The most basic approach, using uninjected arteries, is simply to measure the thickness of the media. In one such study (Barrett, 1963) this was measured at eight equally spaced points round the arterial wall and the mean of the eight measurements taken to represent the artery. Rarely, however, is the medial thickness of any artery based on as many as eight measurements. Depending on whether the measured arteries are cross-sectionally or obliquely cut, medial thickness is usually based on four (ab, cd, ef, gh) or two (ab, cd) measurements (Figure 2.1a). The angle of cut of vessel is one of several points over which workers disagree. Some (e.g. Hasleton et al., 1968; Heath & Best, 1958; Heath & Kay, 1967; Hicken et al., 1965) maintain that only cross-sectionally cut arteries should be measured, and then only if they are virtually circular. Others (e.g. Rabinovitch et al., 1980; Wagenvoort & Wagenvoort, 1965a; Wagenvoort & Wagenvoort, 1982b) insist that oval or obliquely cut arteries should also be included since the shorter of the two axes is representative of the artery. With this in mind there is additional debate on the need for taking measurements in two planes at right angles especially if the artery

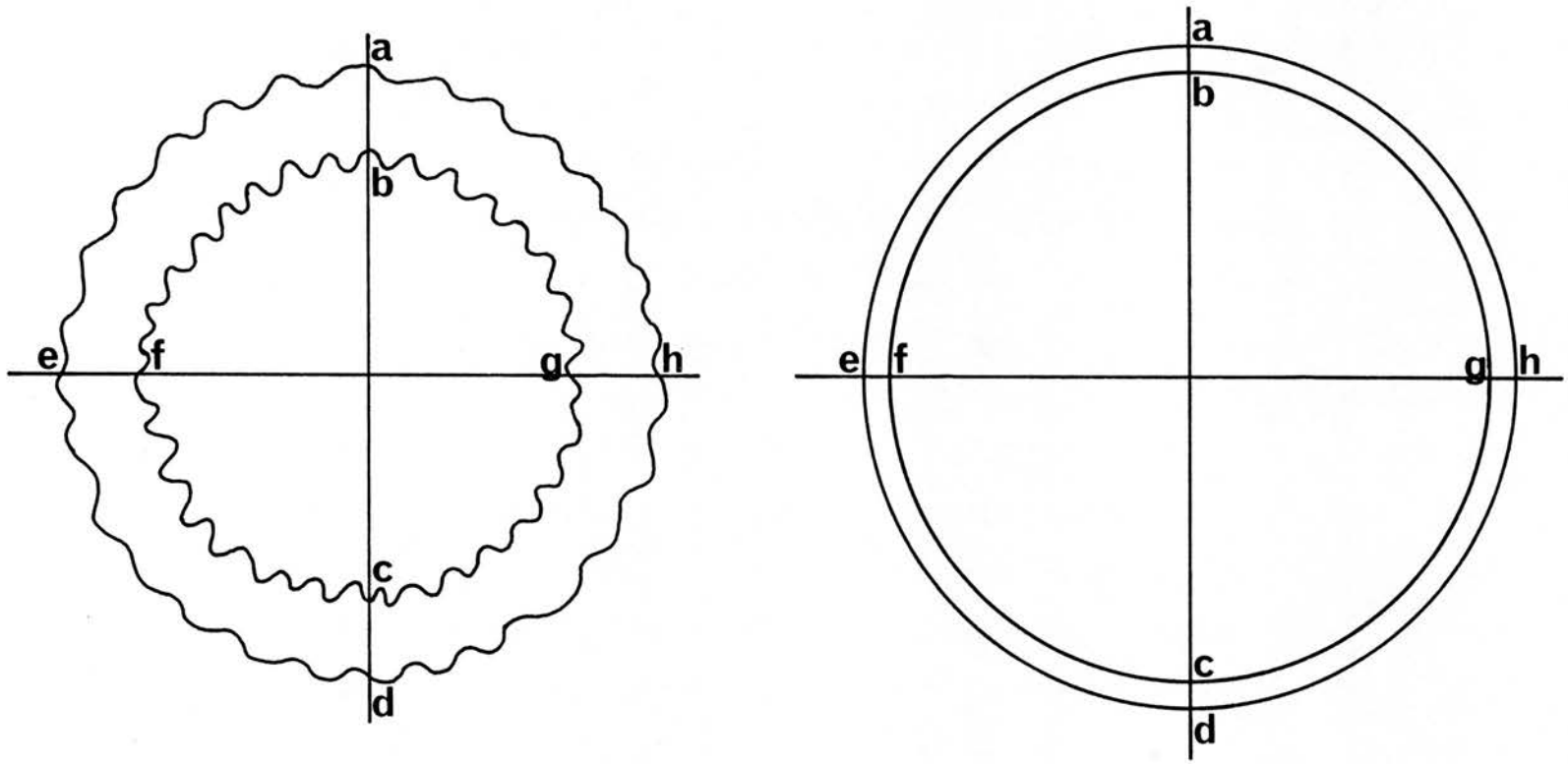


Figure 2.1 Diagrammatic representation of the medial component of an uninjected (a) and injected (b) muscular pulmonary artery.

is circular. Nevertheless, this procedure has been adopted in a number of studies (e.g. Abraham et al., 1971; Heath et al., 1968; Heath et al., 1981; Kay et al., 1967), medial thickness being expressed as the mean of four measurements (ab, cd, ef, gh - Figure 2.1 a). These studies also illustrate what is for most workers the usual approach to the assessment of medial hypertrophy, namely expression of medial thickness in relation to artery size. Here artery size is defined as external diameter and the latter assessed as the mean of two measurements, taken at right angles (ad, eh - Figure 2.1a), of the distance between diametrically opposed points on the external elastic lamina. For each artery medial thickness is then expressed as a percentage of the external diameter, and a mean percentage medial thickness calculated for the subject. Alternatively, arteries may be split into size groups and mean values calculated for each size group (Heath & Best, 1958). A variation on this theme is the use of lumen diameter (mean of bc, fg - Figure 2.1a) rather than external diameter to represent artery size, and expression of the data in the form of a wall to lumen ratio, e.g. Enticknap (1953), Kernohan et al. (1929) and Morlock (1939).

If measurement in two planes is considered unnecessary or obliquely cut arteries are included, then the workers concerned (e.g. Hale et al., 1980; Wagenvoort & Wagenvoort, 1982b; Warnock & Kunzmann, 1977a) express the sum of the medial thicknesses (ab + cd - Figure 2.1a) as a percentage of the external diameter (ad - Figure 2.1a) of the artery. Again, mean values are normally calculated for

the subject but in some studies, e.g. Rabinovitch et al. (1980) and Symchych (1971), the arteries are sub-divided by size.

Two points of general interest emerge from some of the studies mentioned in the preceding paragraph. The first concerns what anatomical boundaries are specified in the measurement of medial thickness, a point most workers neglect to mention but with which one would intuitively expect little variation. In fact medial thickness has been defined as between and including the internal and external elastic laminae (Meyrick & Reid, 1980; Warnock & Kunzmann, 1977a; Warnock & Kunzmann, 1977b), or quite specifically from the inner edge of the external to the outer edge of the internal elastic lamina (Hale et al., 1980). The second point centres on the measurement of external diameter in non-circular arteries. By definition, the shorter diameter is taken as that representing the true size of the artery, but few workers state at what points the measurements are actually made. Exceptions are studies like that of Hale et al. (1980) in which external diameter is clearly defined as the longest diameter between the external elastic lamina in a plane perpendicular to the long axis of the vessel. It has to be presumed that other workers do likewise.

Unfortunately the 'wall thickness' methods for assessing medial hypertrophy in uninjected arteries are fraught with fundamental problems, the most serious of which is the effect of post-mortem collapse or constriction of arteries on the measurements obtained. The seriousness of this problem is evident from the artery illustrated in Figure 2.2. Since collapse/constriction will affect measurements of both medial thickness and artery size, those workers

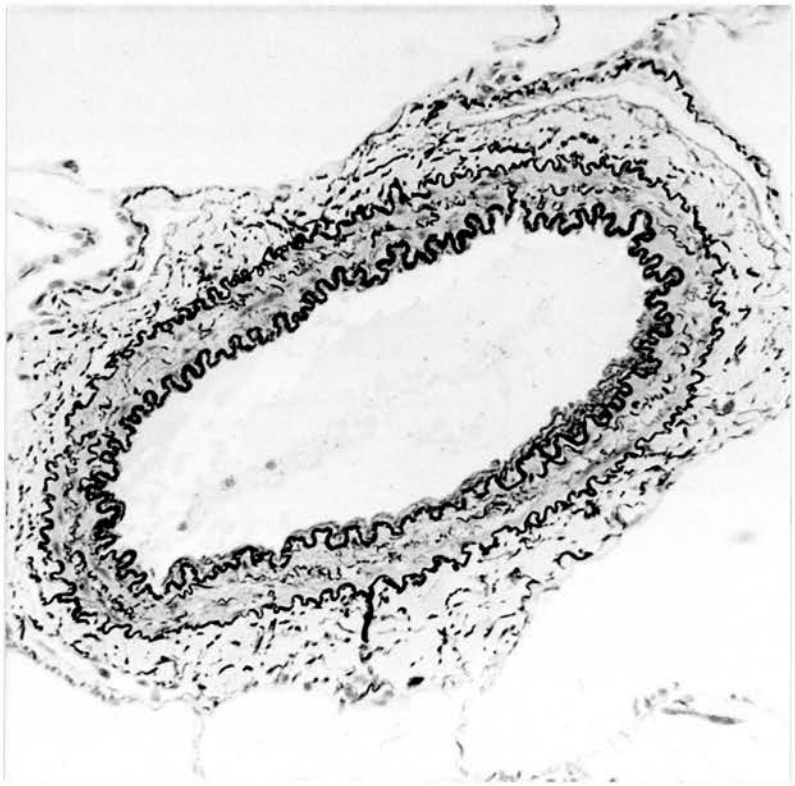


Figure 2.2 A muscular pulmonary artery showing marked constriction/collapse.
x 250
Elastic Stain

who express thickness in relation to artery size, more or less the majority, are unwittingly magnifying the inherent errors. Despite comments from MacWilliam & Mackie -

> "evident that the presence of contractility in an artery at the time it is fixed for microscopical examination, measurements etc. must constitute a source of serious fallacy" (MacWilliam & Mackie (1908).

and further criticisms from a number of workers including Andrus (1936) and Short (1962), these methods are still commonly used on uninjected material.

Problems with the variable collapse or constriction of arteries have prompted workers, determined to use the 'wall thickness' methods for assessing medial hypertrophy, to try other approaches. Still using uninjected material Shapira et al. (1982) landmarked arteries by reference to their accompanying airway, recognising four classes: terminal bronchioles, respiratory bronchioles, alveolar ducts and alveolar walls. For arteries in the latter three classes, they measured medial thickness and external diameter, and calculated mean percentage medial thickness values for each class. This approach only partially overcomes the stated problems.

Perhaps a more sound approach is that adopted by Lynne Reid, amongst others, which is the distension of the pulmonary arteries with an injection medium as described in section 2.1.1 (i). The rationale behind this procedure is simple; complete distension of the arteries should overcome the problems of collapse and constriction and turn external diameter into a meaningful indicator of artery size to which the thickness of the media can be related. As with the uninjected arteries, there are differences between

workers on how to measure and express medial thickness and artery size, and on which arteries ought to be measured. Reid's approach is to measure both cross-sectionally and obliquely cut arteries. For the former, medial thickness is assessed as the mean of four measurements (ab, cd, ef, gh - Figure 2.1b) and external diameter as the mean of two measurements (ad, eh - Figure 2.1b). With obliquely cut arteries the measurements are made only on the shorter of the two diameters. In either case a percentage medial thickness value is calculated for each artery; this equals twice the mean medial thickness expressed as a percentage of the external diameter. Arteries are sub-divided by size and mean percentage medial thickness values calculated for each group. This particular procedure has been used in all studies of pulmonary arteries with which Lynne Reid has been associated in the human (e.g. Hislop & Reid, 1973b; Simons & Reid, 1969; Williams et al., 1979) and in the rat (e.g. Hislop & Reid, 1976; Meyrick & Reid, 1979; Meyrick et al., 1980).

Other workers who have distended arteries with an injection medium have assessed medial hypertrophy in a slightly different way. Kamal & Campbell (1979), for instance, measured only transversely cut arteries. Furthermore they used lumen diameter as an indicator of artery size, as did Short (1966), and expressed medial thickness as a ratio of lumen diameter. Medial thickness has also been expressed as a percentage of lumen diameter by Short & Thomson (1959).

Distension of the pulmonary arteries with an injection medium is not an ideal solution to the problem of artery collapse and constriction; there are problems with the injection method itself, not least of which is knowing what pressure should be used, and also what effect the procedure has on the pulmonary arteries. In view of these problems, many workers have decided that the best solution is to continue to use uninjected material but to seek new methods of measuring the media and artery size, which yield measurements that are more sensible than medial thickness and external/lumen diameter. Without exception these have involved assessment of the area of the medial component, which may be obtained by one of three methods: by mathematical derivation from micrometer measurements of diameter and medial thickness, by planimetry or by point counting.

(iii) Methods involving measurement of medial area

Micrometer measurements of external and internal diameter and medial thickness have been used to calculate medial area, and although the workers concerned may apply different formulae, the end result is the same. This method has been used in several studies of uninjected arteries (e.g. Heath & Best, 1958; O'Neal et al., 1955; Wagenvoort & Wagenvoort, 1982a) but, surprisingly, it has also been used on injected arteries, e.g. Davies & Reid (1970), Short (1966) and Wagenvoort (1960). Differences between workers revolve around the issue of whether only cross-sectionally cut arteries should be measured or whether obliquely cut arteries should also be included. Heath & Best (1958), O'Neal et al. (1955), Wagenvoort (1960) and Wagenvoort & Wagenvoort (1982a) have opted for the former approach,

but in his studies of the systemic circulation (Short, 1966; Short & Thomson, 1959) Short included elliptical vessels taking his measurements on the shorter of the two diameters.

Planimetry is the most commonly used method in which medial area is measured as opposed to calculated. With this method only cross-sectionally cut arteries can be included. Photographs of arteries have been used (Dible, 1964) but the more standard approach is to trace arteries at a known magnification by means of microprojection apparatus (e.g. Barrett, 1963; Furuyama, 1962; Niwa, 1971) or with the aid of a microscope 'camera lucida' (e.g. Cook & Yates, 1972b; Kamal & Campbell, 1979; Naeye, 1965b; Naeye et al., 1974). One extremely important point regarding these methods is how exactly the medial component is delineated when the artery is traced. Regrettably this information is all too frequently lacking. For example, Naeye, in his innumerable studies of the pulmonary vascular bed, has never specified how his tracings are done. Other workers are more precise about the two lines they have used to outline the media. The inner of the two lines may be through the axis of the primary undulations of the internal elastic lamina (Barrett, 1963; Niwa, 1971) but generally the undulations are traced, e.g. Cook & Yates (1972a), Kon (1963) and Suwa & Takahashi (1971). The outer line is always at the junction of the media and adventitia but this is rarely specified as being a tracing of the undulations of the external elastic lamina. An explanation of this probably lies in the fact that many of the studies are of systemic arteries in which the external elastic lamina is either completely absent or extremely thin. In any case, once the tracings are made,

medial area is measured using a planimeter and correction factors applied to take account of magnification.

Point counting is a rarely used method for measuring medial area. In a study of the pulmonary circulation in 1970 (a), Weibel obtained medial area measurements by counting the number of intersections of the media with test-lines of length z.

Area is obviously the most precise measurement of the medial component of pulmonary arteries that can be obtained, and it is indisputably superior to the medial thickness measurement, especially in uninjected arteries. Of the three techniques that have been described for obtaining medial area measurements those of planimetry and point counting are less open to criticism than the use of micrometer measurements of wall thickness and diameter. With the former techniques the area is actually measured but with the latter it is simply estimated. Casting aside the pros and cons of these three methods, however, the important point is that it is relatively easy to measure the medial component of arteries accurately. Unfortunately the same is not true regarding measurements of 'artery size' to which medial area is required to be related. The very difficulties encountered in trying to measure 'artery size', especially in uninjected arteries, are reflected in the diversity of parameters that have been used to describe 'artery size'. These parameters may be conveniently grouped according to common features.

In the first group the parameters used are quite specifically size related, e.g. external diameter (which is obviously inaccurate), or total length of internal elastic lamina. These are arguably the most versatile since they allow data to be obtained for individual arteries.

In the second group the aproach to the assessment of medial hypertrophy is rather different. Instead of choosing parameters which are specifically size related (and difficult to measure) medial area is related to parameters which vary so little between individuals in any particular study that they can effectively be used as a base-line. The parameters included in the second group are artery position, e.g. type of accompanying airway, which is acceptable, or facets of artery structure, e.g. cross-sectional area of intima or intimal nuclei, which is less acceptable because of the assumption of a normal intima, often without evidence.

In the third group the parameter to which medial area is related is unit area of lung tissue.

The parameter of choice depends almost entirely on the objectives of the study in question, whether data for the individual artery are to be obtained or data for the lung as a whole. For example, the group 3 parameter, unit area of lung tissue, would be unsuitable in the former situation but ideal in the latter.

(iv) Parameters to which medial area is referred in the assessment of medial hypertrophy

Unit area of lung tissue. The principle behind this as a means of assessing medial hypertrophy is quite straightforward, the amount of arterial muscle is measured in a number of tissue sections and expressed per unit area of lung tissue. By comparison with control values it is possible to determine whether or not medial hypertrophy occurs in certain disease states. This technique necessitates measuring the medial area of all arteries within a histological section, which is difficult, and several different approaches have been tried. Wagenvoort (1960), for example, started by classifying the circular arteries into two size groups less than 100μm and greater than 100μm in external diameter. For each artery he measured diameter and medial thickness which were used to calculate the area of the media. Mean medial area values were then determined for arteries measuring less than and greater than 100μm in diameter. Using a planimeter the surface area of each histological section was measured from which the surface area of the great bronchi and vessels was deducted. Counts were made of the number of arteries (circular and oblique) measuring less than 100μm and greater than 100μm in diameter, and from this the number of each calculated per sq cm of lung parenchyma. Using the mean medial area values the total cross-sectional area of the media of arteries in both categories was found, and their sum used as an index of the amount of vascular tissue per sq cm of lung parenchyma. Wagenvoort has applied this technique for assessing medial hypertrophy in pulmonary hypertension (Wagenvoort, 1960) and in primary pulmonary

hypertension (Wagenvoort & Wagenvoort, 1970). In the former study the pulmonary arteries were distended by an injection medium. Also working with distended arteries Davies & Reid (1970) used a variation of Wagenvoort's technique for assessing the amount of muscle per unit area of lung tissue. First of all they restricted their assessment to arteries measuring less than 200μm on the grounds that the smaller arteries are the first to show any changes if medial hypertrophy occurs. Arteries measuring less than 200μm were sub-divided by external diameter into five groups: 25-50, 50-75, 75-100, 100-150 and 150-200μm, and the number of cross-sectional or oblique arteries in each group counted. Using micrometer measurements of diameter and medial thickness the medial area of an 'average' (subjective assessment) artery in each size range was calculated and multiplied by the number of arteries in that range. Allowance was made for partially-muscular arteries by assuming that the average partially-muscular artery has muscle about 50% of its circumference. The total area of muscular media of all arteries up to 200μm in external diameter was then calculated per unit area of lung tissue.

The technique of assessing medial area per unit area of lung tissue has several disadvantages apart from the disadvantage of not being able to obtain data for the individual artery. Firstly, sampling has to be very strictly controlled. Secondly, the methods used to quantitate total medial area provide only an estimated value since not all arteries are measured, and for those that are, medial area is calculated from micrometer measurements of diameter and wall thickness.

Type of accompanying airway. Appreciating the problems of trying to find a reliable measure of artery size in uninjected arteries, O'Neal et al. (1955) divided arteries into two classes, those lying adjacent to the origin of respiratory bronchioles and those lying adjacent to peripheral respiratory bronchioles. From micrometer measurements of diameter and medial thickness the medial area of each artery was calculated and mean values obtained for the two classes of artery.

Criticisms of this technique relate to the fact that medial area is estimated and not measured, and also to the disparity between the branching pattern of the pulmonary arterial tree and bronchial tree as noted by Elliott & Reid (1965).

Cross-sectional area of intima plus internal elastic lamina. The use of this parameter as a base-line for comparing medial area measurements in the normal and diseased states is peculiar to one worker in the field of pulmonary arteries, Naeye, who used it only in the very earliest of his studies (Naeye, 1961a; Naeye, 1961b). Measurement of the area of intima plus internal elastic lamina is obtained as for medial area by planimetry (see section 2.1.2. (iii)).

Total area of arterial intimal nuclei. In Naeye's studies this parameter very quickly took precedence over the cross-sectional area of intima plus internal elastic lamina as the base-line to which measurements of medial area were referred. In fact, it has been used in the majority of his reported studies of pulmonary arteries

(e.g. Naeye, 1966; Naeye, 1967a; Naeye, 1969; Naeye et al., 1971) and also systemic arteries (Naeye, 1967b). Again the total area of arterial intimal nuclei is obtained by planimetry.

One of the major drawbacks of Naeye's techniques is that they cannot be used in cases where any intimal sclerosis is present and since this is common even in apparently normal adults their usefulness is limited.

External diameter. This is one of the few size specific parameters of pulmonary arteries to which medial area has been related. Unfortunately it is a very poor indicator of artery size in uninjected arteries as it varies with the degree of post-mortem collapse or constriction present. Nevertheless, some workers, e.g. Heath & Best (1958) have sub-divided arteries by external diameter and calculated mean medial areas for each group. Apart from the criticism regarding the use of diameter in uninjected arteries, this study may be faulted because the medial area was calculated from micrometer measurements of diameter and medial thickness, and not measured.

Total length of the internal elastic lamina. Several workers have concluded that this parameter is the only sensible indicator of artery size in uninjected arteries because it satisfies the essential criterion of being unaffected by collapse or constriction. A further advantage of having such a direct measure of artery size is that in the assessment of medial hypertrophy arteries can be studied on an individual basis by relating medial area to length of

internal elastic lamina. This is a considerable advantage in situations where vascular abnormalities are patchy in distribution.

With the exception of Weibel (1970a) all workers using this parameter have measured it from tracings of arteries. In their studies of systemic arteries Cook & Yates (1972b) and Kamal & Campbell (1979) used a rotameter to obtain the total length of the internal elastic lamina. Others, studying either systemic (Furuyama, 1962; Suwa & Takahashi, 1971), or pulmonary arteries (e.g. Honda, 1967; Yamaki & Horiuchi, 1979; Yamaki & Tezuka, 1976) have measured it by attaching thin thread on to their tracings and measuring the length used. Weibel's approach is completely different, the length of the internal elastic lamina is obtained directly from the histological section from counts of the number of intersections of the structure with test-lines of length z (Weibel, 1970a).

The total length of the internal elastic lamina would indeed appear to be an almost ideal indicator of artery size. By theoretically unwrinkling the internal elastic lamina it is possible to visualize the artery in its uncollapsed/unconstricted state. Furthermore, all arteries are reduced to the same state. However, there do seem to be difficulties in measuring the length of the internal elastic lamina insofar as the majority of methods used are extremely time-consuming and tedious. This is an area with scope for improvement.

(v) Summary

Medial hypertrophy may be assessed in one of two ways, by determining whether or not there is an extension of arterial muscle into previously non-muscularised vessels, or by measurement of existing muscular arteries. The 'wall thickness' methods are those most commonly used for the latter. These methods are not really suitable for use on uninjected arteries because the measurements of medial thickness and artery size vary with the degree of post-mortem collapse or constriction present. Distension of arteries with an injection medium may sound a simple solution to this problem but there are problems with the injection method itself. Attempts to find more valid methods of assessing both the media and artery size have invariably involved measurement of medial area. In contrast, a variety of parameters have been used as 'base-lines' to which medial area is referred. However, only one of these, the total length of the internal elastic lamina, is both size specific and unaffected by collapse or constriction of the artery.

2.1.3 Methods for Assessing the Intimal Component of Pulmonary Arteries

Although there have been many quantitative studies of the pulmonary vasculature, especially the muscular pulmonary arteries, the emphasis has always been on measurements of the media; few studies have included any quantitation of the intimal component. There are two likely explanations for this. Firstly, there has been a tendency to underestimate the potential importance of intimal changes as a cause of increased pulmonary vascular resistance and

hence pulmonary hypertension. The second explanation lies in the nature and distribution of intimal changes which occur in pulmonary arteries. In most disease states these changes are patchy, rarely affecting the entire circumference of an artery to a uniform extent (Harris & Heath, 1977; Wagenvoort & Wagenvoort, 1977). This is illustrated in Figure 2.3. Furthermore, variable numbers and different size groups of arteries may be affected (Harris & Heath, 1977; Wagenvoort & Wagenvoort, 1977). These factors make quantitation of the intima rather less than straightforward.

The following description of methods that have been used for assessing the intimal component of pulmonary arteries is ordered as for the media beginning with the simplest and working through to the most sophisticated.

(i) Subjective description

This sort of approach is prevalent in studies where assessment of medial changes has been the main objective. Normally intimal thickening or abnormality are the factors commented upon, e.g. Ryland & Reid (1975), Semmens (1970) and Semmens & Reid (1974).

(ii) Proportion of arteries affected

Here the 'subjective description' approach is taken one step further; the presence of intimal fibrosis/proliferation is recorded and expressed as the percentage of vessels affected. Depending on the workers involved, this may refer to the total artery population (Brenner, 1935b; Warnock & Kunzmann, 1977a; Warnock & Kunzmann,

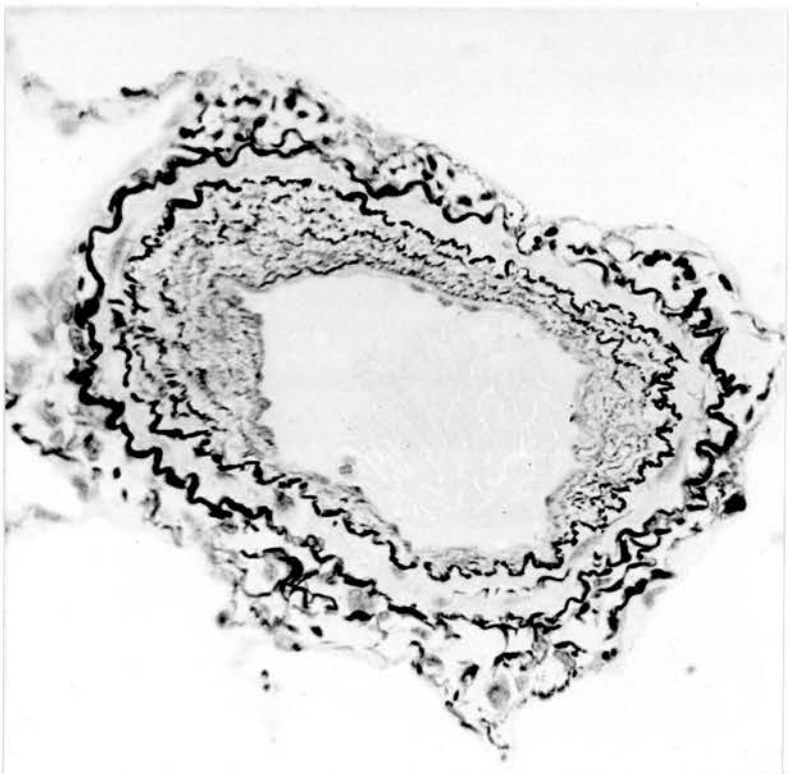


Figure 2.3 A muscular pulmonary artery showing an irregular layer of intimal thickening.
x 450
Elastic Stain

1977b), or it may be only those arteries associated with alveolar ducts and alveolar walls (Shapira et al., 1982).

(iii) Grading methods

Standard grading methods have been developed by a number of workers in an attempt to simplify description of structural changes in the pulmonary arteries. As one might expect, these grading systems encompass changes in the media as well as in the intima.

The system of Heath & Edwards (1958) describes six grades of structural changes in the pulmonary arteries of which grades 1-4 include specific intimal changes. This classification system was devised with congenital cardiac septal defects in mind and it has been used by Heath et al. (1958) in a study of ventricular and atrial septal defects.

Wagenvoort, who has also attempted to classify pulmonary vascular disease (1973), is of the opinion (1981) that the Heath-Edwards classification is of limited general value and should only be used in congenital cardiac disease with a left to right shunt or in classical primary pulmonary hypertension. In view of these comments Symchych's (1971) use of the Heath-Edwards system in a study of pulmonary hypertension in cystic fibrosis seems rather inappropriate.

A fairly recent grading system is that devised by Yamaki & Tezuka (1976) and used in further studies by Yamaki & Horiuchi (1979), Yamaki & Tezuka (1979) and Yamaki & Wagenvoort (1981). Four

grades of structural changes are described in this grading system, the first three of which relate to the intima. Grade 4 involves destruction of the media.

Subjective grading methods, unlike the standard grading methods, are concerned more with the amount of intimal change present rather than the type of change present. For instance, intimal changes have been graded as absent, slight, moderate or severe (James & Thomas, 1963) or on a scale of + through to ++++ (Brenner, 1935b). Wagenvoort, who believes the type of intimal fibrosis to be critical, especially with regard to evaluation of biopsy specimens, has tried combining grading of type and amount of intimal change. In three studies of the pulmonary vasculature (Wagenvoort, 1980a; Wagenvoort et al., 1967a; Wagenvoort et al., 1967b) he has graded the pulmonary arteries for, amongst other features, concentric laminar intimal fibrosis and eccentric intimal fibrosis, each on a scale of - (absent), + (mild), ++ (moderate) and +++ (severe).

(iv) Methods involving measurement of the intimal component

The most basic method involves measuring the thickness of the intimal layer but, in view of the often irregular distribution of intimal abnormality round an artery wall, decisions have to be made on where the measurements should be taken. Anderson et al. (1973) chose to measure the thickness of the intima at the site of least intimal thickening from the inner edge of the internal elastic lamina, admitting that by so doing their method would underestimate the severity of intimal disease.

Normally when the thickness of the intimal component is measured it is set in relation to the size of the artery, for which diameter is the parameter normally chosen. These particular methods are essentially the same as the 'wall thickness' methods for assessing medial hypertrophy, which were described in section 2.1.2 (ii). As with the latter methods there are differences between workers with respect to what arteries should be measured, how artery size should be defined, and how the data should be expressed.

Some workers, e.g. Heath et al. (1981) and Kay & Smith (1973), have measured only transversely sectioned arteries and taken measurements in both planes. With this procedure, intimal thickness is assessed as the mean of four measurements (bc, de, hi, jk - Figure 2.4) taken at equally spaced points round the artery lumen. Heath et al. (1981) defined artery size as internal diameter, assessing it as the mean of two measurements (be, hk - Figure 2.4) taken on the internal elastic lamina. For each artery intimal thickness was expressed as a percentage of internal diameter and a mean value calculated for all arteries measured. In contrast, Kay & Smith (1973) used external diameter rather than internal diameter as an indicator of artery size but otherwise their method of expressing intimal thickness was similar.

Other workers, e.g. Hale et al. (1980), Wagenvoort & Wagenvoort (1965a) and Wagenvoort & Wagenvoort (1973) have chosen to include obliquely sectioned arteries and taken measurements on the shorter of the two diameters only. In these three studies intimal thickness was assessed as the sum of the intimal thicknesses on both sides of

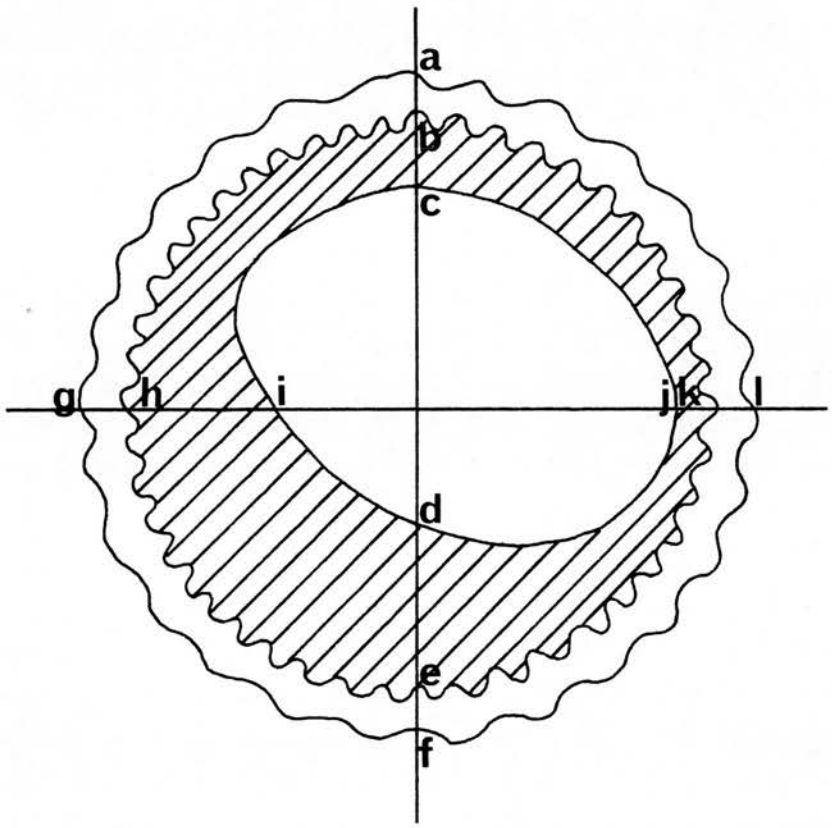


Figure 2.4 Diagrammatic representation of a muscular pulmonary artery; the intimal component is the shaded area.

the artery (bc + de - Figure 2.4), expressed as a percentage of the external (Hale et al., 1980) or internal diameter (Wagenvoort & Wagenvoort, 1965a; Wagenvoort & Wagenvoort, 1973). Appreciating the variation in intimal thickness round an artery wall, Brenner (1935b) expressed intimal thickness as a percentage of external diameter but also quoted the range of extremes seen, both in the individual artery and in the subject.

The 'intimal thickness' methods for assessing the intima of pulmonary arteries may be criticised for the same reasons as the 'wall thickness' methods for assessing the media; the measurements produced do not accurately reflect either the amount of intimal change or the size of artery affected, since they vary with the degree of constriction present. Few workers have attempted to use these methods on arteries distended by an injection medium. Warnock & Kunzmann (1977a) are some of the few but they assessed the total thickness (media plus intima) of the artery wall rather than just the intima itself.

A more accurate method of assessing intimal abnormality would be to measure intimal area. This has been done in a study of the systemic circulation (Dible, 1964); Dible very cleverly used the area measurements (obtained by planimetry) to calculate the thickness of the intimal layer, assuming it to be evenly distributed round the artery wall. Unfortunately he then spoilt the study by not taking post-mortem collapse or constriction of arteries into account in his assessment of artery size. The only other worker to have measured intimal area (again by planimetry) is Naeye; (e.g.

Naeye, 1961a; Naeye, 1961b). However, to Naeye the area of the intima was of no interest in itself; it was merely used as a base-line to which he could relate his measurements of medial area.

(v) Summary

Relatively little work has been done on assessing the intimal component of pulmonary arteries compared with the amount of work done on the media. To some extent this is reflected in the crudity of the methods used, the most frequent being simple grading methods of either the type or amount of intimal abnormality. Measurements, where taken, are usually of intimal thickness expressed in relation to artery diameter. Since these methods have almost invariably been applied to arteries which have not been distended by an injection medium the measurements of both intima and artery size vary with collapse or constriction of the artery. Area measurements would greatly improve the accuracy of assessment of intimal abnormality but very few workers have measured this. Furthermore, no attempts have been made to find a sensible indicator of artery size to which measurements of the intima could be related.

2.1.4 Other Factors of Importance in Quantitative Studies of Pulmonary Arteries

In any quantitative study of the medial or intimal component of pulmonary arteries there are factors other than the method of tissue preparation and the measuring technique used, which are important. Three factors in particular spring to mind: the artery population

measured, the number of arteries measured, and the repeatability of the measurements obtained.

(i) The artery population measured

Obviously the artery population available for measurement is dependent on the sampling procedure used in the tissue preparation process. What arteries are measured, however, depends very much on whether any exclusion criteria are applied. Ignoring the exclusion criterion of obliquely cut arteries, which is often imposed by the measuring technique itself, the commonest exclusion criteria relate to type or size of artery. For instance, in the assessment of medial hypertrophy Heath et al. (1968) measured only those arteries with an external diameter of less than 1000μm. Normally when size dependent exclusion criteria are applied the limits are set much lower than this. Arteries less than 400μm (Lodi & Viswanathan, 1974; Matsubara et al., 1984), less than 300μm (Hasleton et al., 1968; Heath & Kay, 1967) and even less than 150μm (Larrabee et al., 1949; Naeye & Laqueur, 1970; Naeye et al., 1971) in external diameter are quite commonly the only arteries measured. In the assessment of intimal abnormality some studies, e.g. Hale et al. (1980) and Hasleton et al. (1968) have included only those arteries measuring less than 500μm or 300μm in diameter respectively. In instances such as these the workers are limiting themselves to study of the smaller muscular pulmonary arteries and arterioles on the grounds that these are the most reactive of the pulmonary vessels and will therefore be the first to show any medial or intimal changes.

For some workers the presence of intimal abnormality will preclude the media of an artery from being measured. With Naeye this criterion is necessarily imposed by the technique he uses for assessing medial hypertrophy (see section 2.1.2 (iv)) but for others (Goodale & Thomas, 1954; Yamaki & Tezuka, 1976) it is imposed in an effort to avoid secondary thinning of the media brought about by extensive intimal changes.

Any other exclusion criteria are likely to be specifically associated with the measuring technique used. For example, where artery size is defined in terms of total length of internal elastic lamina one would expect a well-defined internal elastic lamina to be essential for measurement.

So, in quantitative studies of the media and intima of pulmonary arteries decisions have to be made on what arteries should be measured. In addition care should be taken in the interpretation of results if exclusion criteria other than size or angle of cut of artery are applied.

(ii) <u>The number of arteries measured</u>

To a large extent this is governed by the number of tissue blocks selected for study and the exclusion criteria applied, to the latter of which is linked the sophistication of the technique used. Over and above this, however, workers may adopt one of two approaches. Within the limits set they may measure all available arteries, alternatively they may decide to measure a minimum number of arteries and take extra tissue blocks if required. There is

consequently a great deal of variation in the number of arteries on which workers base an assessment of medial hypertrophy or intimal abnormality. With regard to the former, as few as five arteries have been measured, the first five to be encountered (Larrabee et al., 1949). Naeye has also tended to measure very few arteries, less than 10, in some of his studies of the pulmonary vasculature (Naeye, 1966; Naeye, 1967a; Naeye, 1969). At the other extreme, 45-50 arteries per lobe have been measured (Warnock & Kunzmann, 1977a; Warnock & Kunzmann, 1977b), although 50 per case is a more common maximum (Wagenvoort & Wagenvoort, 1970; Wagenvoort & Wagenvoort, 1982b), those of the latter studies being randomly selected. The norm, however, seems to be somewhere between 10 and 50 per case, generally 20-25, e.g. Naeye (1961a), Wagenvoort & Wagenvoort (1982a) and Wagenvoort et al. (1974).

In view of the often patchy distribution of intimal abnormality it is even more important to ensure that an adequate number of arteries is measured when assessment of intimal abnormality is the main study objective.

(iii) Repeatability of measurements

This is an area of immense importance in quantitative studies of the pulmonary vasculature. If a technique is to be considered practicable then, amongst other factors, it has to give good reproducibility of measurements by the individual observer, and also by different observers. If this cannot be shown then doubts must be cast on the technique itself and, more important, on the measure-

ments produced by it. In their enthusiasm to study medial and intimal changes in pulmonary arteries most workers have tended to ignore this very important point. In fact, of all the references quoted in this chapter, mention of either intra- or inter-observer repeatability of measurements is made in only three studies. Hale *et al.* (1980) claim to have tested intra-observer variability and Hunter *et al.* (1974) state that their results were repeatable by one individual, and that there was good agreement between the two individuals who did the measurements. However, only Rabinovitch *et al.* (1980) give precise details of how their intra- and inter-observer repeatability tests were carried out.

(iv) Summary

Apart from the method of tissue preparation and the measuring technique used, a further three factors are of importance in quantitative studies of the pulmonary vasculature, these are: the arteries selected for measurement, the number measured, and the reproducibility of the measurements obtained by the technique used.

2.2 AIMS

The main aim of Chapter 2 was to develop a method for measuring certain parameters of muscular pulmonary arteries, notably medial area, intimal area and total length of internal elastic lamina, using a semi-automatic measuring system (a digitiser). Following on from this general aim there were a number of specific aims; these were:-

1. To test the reproducibility of measurements obtained using the described technique.
2. Having determined the criteria by which an artery would be considered measurable by the described technique, to test the stringency of these criteria.
3. To determine whether or not the measured arteries are a representative sample of the total muscular pulmonary artery population.
4. To investigate the relationship between medial area and artery size (defined in terms of length of internal elastic lamina).
5. To use this relationship to determine the effect of various tissue preparation procedures on measurements of muscular pulmonary arteries. The tissue preparation procedures to be investigated were:-

   (i) Arterial distension with an injection medium. To be done by comparing injected and uninjected muscular pulmonary arteries.

   (ii) Tissue fixation. To be done by comparing muscular pulmonary arteries from uninflated, routinely inflated and constant pressure inflated lungs.

(iii) Tissue processing. To be done by comparing muscular pulmonary arteries from paraffin and glycol methacrylate embedded and sectioned tissue.

6. To investigate the relationship between area of intima and artery size (length of internal elastic lamina), and to determine the best way of expressing patchy intimal abnormality.

7. To establish a method for maximising the data obtainable from those arteries not considered measurable by the described technique.

2.3 MATERIAL AND METHODS

2.3.1 The Subjects

Thirteen subjects were included in the technique validation studies. Information on the age, sex and smoking history of these subjects is given in Table 2.1, together with details of the relevant cardio-pulmonary pathology.

Fairly extensive use was made of post-mortem material that had been prepared prior to the start of the present study in 1981; this material, the histological sections of subjects 1-7 inclusive, was provided by Dr David Lamb of the Department of Pathology, University of Edinburgh. These seven subjects were selected for inclusion in this chapter for the following reasons. As a group they covered a variety of disease states and included what might be considered a 'normal', i.e. no specific abnormalities; consequently the muscular pulmonary artery populations showed a wide range of features, making it an easy task to select a sample on which to test the reproducibility of measurements (see section 2.2 Aim 1). The variety of disease states also made the group ideal for another purpose, which was the investigation of the relationship between area of intima and artery size (see section 2.2 Aim 6).

For four of the seven subjects, 1-4 inclusive, the tissue preparation procedure had included distension of the pulmonary arteries of one lung with an injection medium; as such, they were suitable material for determining the effect of this procedure on measurements of the media and artery size (see section 2.2 Aim

Table 2.1 Details of age, sex, smoking history and relevant cardio-pulmonary pathology for the 13 study subjects.

Subject Number	Age	Sex	Smoking History	Cardio-pulmonary pathology
1	56	M	Smoker	COLD*, marked RVH+
2	55	M	Smoker	COLD, no RVH
3	67	M	Smoker	COLD, marked RVH
4	64	M	Unknown	Rheumatic heart disease
5	59	M	Smoker	No specific abnormalities
6	67	M	Unknown	Atrial septal defect
7	24	M	Unknown	Pulmonary sequestration
8	62	M	Unknown	No specific abnormalities
9	69	F	Unknown	Mild LVH#
10	81	M	Unknown	No specific abnormalities
11	80	M	Unknown	No specific abnormalities
12	51	F	Smoker	Small peripheral lung carcinoma
13	59	M	Smoker	Small peripheral lung carcinoma

* COLD = Chronic obstructive lung disease

+ RVH = Right ventricular hypertrophy

LVH = Left ventricular hypertrophy

5 (i)). Finally, this particular group of four subjects was considered an acceptable group on which to test the repeatability of selection of arteries considered measurable, and on which to compare the measured arteries with the total population (see section 2.2 Aims 2 and 3 respectively).

Some of the aims of the chapter required new material to be found; this material, from subjects 8-13 inclusive, was obtained from the Royal Infirmary of Edinburgh or the City Hospital, Edinburgh during the period 1980-1983. The lungs of subjects 8-11 were obtained post-mortem, those of subjects 12 and 13 were resection specimens.

In order to study the effect of different tissue fixation procedures on the pulmonary arteries (see section 2.2 Aim 5 (ii)), it was considered sensible to use lungs which showed minimal disease. Since the lungs could not be examined until after the tissue fixation procedures were complete, assessment of disease was based on the clinical history of the patient and external examination of the lungs. As pairs of lungs with minimal disease became available they were incorporated into the study; in all four pairs were obtained (subjects 8-11).

In addition to being used to study the effect of different tissue fixation procedures on pulmonary arteries the lungs of subjects 10 and 11 were used to study the effect of different tissue embedding media (see section 2.2 Aim (5 (iii)), and also to test ways of maximising the data obtainable from muscular pulmonary

arteries (see section 2.2 Aim 7). Also used for these two purposes was the lung of subject 12.

Subject 13 was included in the study because histological examination revealed that extensive and severe intimal abnormality was present in the muscular pulmonary arteries. This subject was, therefore, considered a useful addition to those already selected for study of the relationship between area of intima and artery size (see section 2.2 Aim 6).

To assist the reader a summary of the subjects included in each of the main areas investigated is given in Table 2.2.

2.3.2 Distension of Pulmonary Arteries

At autopsy, usually within 24 hours of death, both lungs were removed intact leaving as good a length of the main bronchi and pulmonary arteries as possible. One lung from each pair was then warmed by immersion in a water bath maintained at 37°C. This step was extremely important as it helped avoid early solidification of the injection medium. After tying a cannula into the main pulmonary artery, a previously prepared barium-gelatin mixture at a temperature of 60°C was injected using a hand-held syringe with a three way tap connected to the manometer of a blood pressure machine. The injection pressure, in excess of 100cm H_2O, was maintained for several minutes after which the cannula was clipped; hypertensive pressures were used in line with other workers, e.g. Reid (1967), Doyle *et al.* (1957) and Warnock & Kunzmann (1977a) in

Table 2.2 Summary of the subjects included in each of the main areas investigated.

Area of Investigation	Subject Number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Reproducibility of measurements	*			*	*	*	*						
Reproducibility of artery selection	*	*	*	*									
Comparison of 'digitisable' arteries and total population	*	*	*	*									
Relationship between medial area and artery size	*	*	*	*	*	*	*	*	*	*	*	*	*
Comparison of :injected and uninjected arteries	*	*	*	*									
:uninflated and routinely inflated tissue								*	*				
:routinely inflated and constant P* inflated tissue										*	*		
:GMA+ and paraffin embedded tissue										*	*	*	
Relationship between intimal area and artery size	*			*	*	*	*						*
Methods for maximising measurements										*	*	*	

* P = Pressure

+ GMA = Glycol methacrylate

order to overcome post-mortem contraction and to produce complete distension of arteries and arterioles.

The barium-gelatin injection medium used was that described by Reid (1967). It was prepared by dissolving 50g of gelatin powder in 200ml of warm water to which 500ml of 'Micropaque' (obtained from Damancy & Co.) was slowly added, whilst stirring. Penetration down to the level of small pulmonary arterioles was achieved but the medium did not enter the capillaries.

One lung from each of four subjects (1-4, Table 2.1) was prepared in this manner by Dr David Lamb. Upon completion of the injection procedure both lungs were immediately fixed by inflation with formol saline according to the routine method described in the following section. Tissue fixation resulted in solidification and cooling of the injection medium.

2.3.3 Tissue Fixation

Simple immersion in formol saline was the method of tissue fixation for the two lungs (subjects 8 and 9) which were not inflated. The fresh lungs were sliced and blocks of tissue randomly selected (see section 2.3.4); these blocks were fixed in jars of formol saline for at least one week.

For routine inflation formol saline was allowed to run into the main bronchus through plastic tubing from a bottle placed approximately 45cm above the work-bench; the formol saline was allowed to run in until the pleura was firm with rounded edges. Fixation was then allowed to continue for at least one week by

placing the lung in a basin of formol saline, covered to prevent drying.

This method of tissue fixation was the one most commonly used; at least one lung from each of the 13 subjects was thus fixed.

For constant pressure inflation, apparatus similar to that described by Heard et al. (1967) was used. The apparatus is illustrated in Figure 2.5. Cannulas were inserted into the main bronchi of the lungs and formol saline instilled at a constant pressure of 25-30cm for four days. The 25-30cm refers to the height difference between the base-line level of formol saline in the lower tank and the level in the formol saline reservoir. As the level of formol saline in the lower tank rose beyond the base-line, the excess was pumped through the level maintainer back into the reservoir.

As with routine inflation, fixation was continued for a further period by allowing the lung to float in a basin of formol saline, which was covered to keep the lung moist.

Two lungs (from subjects 10 and 11) were fixed using the constant pressure inflation apparatus.

In all, three people from the Department of Pathology, University of Edinburgh carried out the tissue fixation procedures described in this section. Dr David Lamb fixed the lungs of subjects 1-7, Dr Lesley Turnbull, subject 8 and Alec McLean, subjects 9-13.

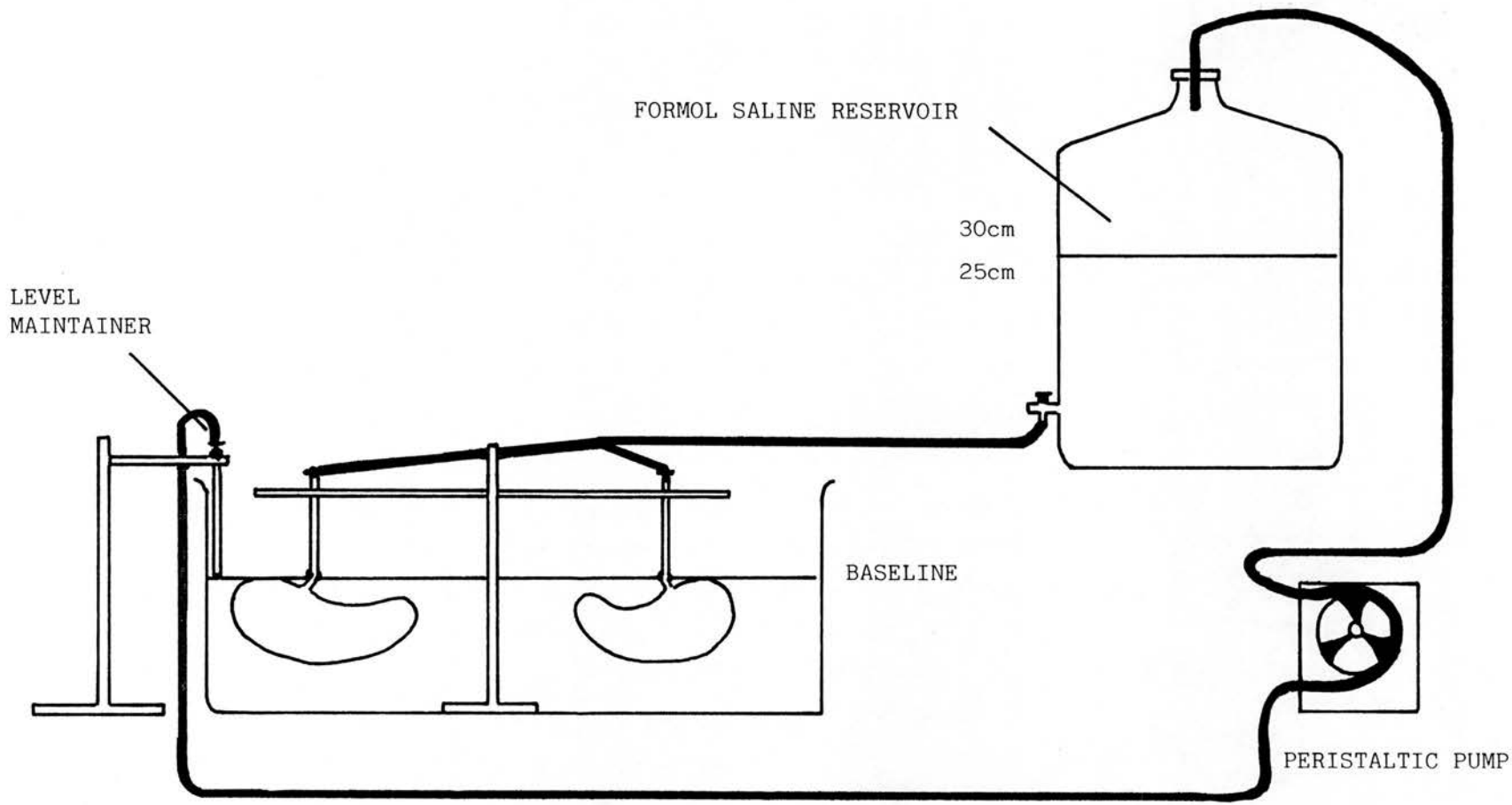


Figure 2.5 The apparatus used for constant pressure inflation of lungs.

2.3.4 Selection of Tissue Blocks

Following fixation, lungs were sliced at 1cm intervals in the sagittal plane and tissue blocks selected. In general a total of 12 blocks was taken from each lung, six from each of the upper and lower, or lower plus middle lobes. Two methods of selection were utilised. In the case of subjects 1-8 inclusive, representative blocks were taken from throughout each lung avoiding large structures; these blocks measured approximtely 2.54cm x 2.54cm x 1.27cm (1" x 1" x $^{1}/_{2}$"). Although 'randomly' selected the decision on where to take the tissue blocks was subjective.

With subjects 9-13 the tissue blocks were selected in true random fashion using a random number table and template. To avoid the possibility of selecting adjacently sited blocks the random sampling was stratified as described by Dunnill (1964). The twelve blocks were a standard size 1.91cm x 1.91cm x 0.64cm ($^{3}/_{4}$" x $^{3}/_{4}$" x $^{1}/_{4}$") and were taken from the two most lateral slices of each lung, six from each slice. In the case of subjects 12 and 13 the upper lobe of each lung was excluded because it contained a small peripheral carcinoma; the 12 tissue blocks therefore came from the lower lobe only.

For each of the 13 subjects the person who fixed the lung(s) (see section 2.3.3) also selected the tissue blocks.

2.3.5 Tissue Embedding

Two tissue embedding media were utilised, paraffin wax and the acrylic resin, glycol methacrylate.

(i) Paraffin wax

Dehydration of tissue prior to paraffin wax embedding was effected by processing the samples through an ascending alcohol series into xylene. Tissue samples were then embedded in paraffin wax (melting point 56°C) in a vacuum oven. The description of this procedure has been kept brief simply because it is so well-known. Full details may be found in any textbook on histological technique, e.g. Carleton's Histological Technique, 4th Edition (Drury & Wallington, 1967).

(ii) Glycol methacrylate

For those tissue samples to be embedded in glycol methacrylate the histokinette schedule was as follows:-

1.	10% ethanol	2 hours
2.	20% ethanol	"
3.	30% ethanol	"
4.	50% ethanol	"
5.	80% ethanol	"
6.	64 O.P. spirit	"
7.	64 O.P. spirit	"
8.	74 O.P. spirit	"
9.	74 O.P. spirit	"

10. acetone "

11. absolute ethanol "

12. absolute ethanol "

The following glycol methacrylate (GMA) solutions were used:-

Solution A

2 - hydroxyethyl methacrylate	400ml
2 - butoxyethanol	40ml
benzoyl peroxide	3.5ml

The peroxide is added last and the solution stirred automatically for two hours; the volume of peroxide may be varied.

Solution B

polyethylene glycol	8ml
N.N. Dimethylaniline (carcinogen)	1ml

Embedding solution

Solution A	42ml
Solution B	1ml

Following the histokinette schedule tissue samples were transferred into specimen bottles containing GMA solution A using a fume cupboard. The tissue samples were impregnated in two changes of GMA solution A, each for 24 hours. Negative pressure was found to be helpful. At this point the tissue samples were ready for embedding. A selected quantity of GMA solution A was poured into a 16oz bottle and solution B prepared using disposable 5 and 1ml syringes. The GMA solution A in the specimen bottles was discarded. Solution B was introduced into solution A in the 16oz bottle in a

ratio of 1:42 and the resultant embedding solution mixed by hand. The specimen bottles and plastic moulds were then charged with the GMA embedding mixture and the bottles rotated on a roller for five minutes. Next, the tissue blocks were transferred to plastic moulds and orientated. When the GMA embedding mixture became tacky the polymerising reaction was slowed down by placing the moulds on crushed ice for a minimum of one hour. The moulds were then peeled off and the blocks hardened at 60°C.

This method for embedding tissue in glycol methacrylate is a variation of that described by Sims (1974).

2.3.6 Tissue Sectioning

Tissue blocks embedded in paraffin wax were sectioned at 5μm. Those embedded in glycol methacrylate were sectioned at 3μm according to the method of Sims (1974) on a Reichert Jung Autocut using a tungsten carbide knife.

2.3.7 Staining of Tissue Sections

When studying the pulmonary vascular bed the most useful stains are those which clearly define the elastic tissue. For paraffin embedded tissue either Weigart's elastic stain with a van Gieson counterstain, or Miller's elastic stain were used. A modification of Verhoeff's elastic stain was used for glycol methacrylate embedded tissue. The staining procedure used was similar to that described by Musto (1981). Tissue sections were mordant overnight in Bouin's fluid. After washing to remove the yellow colour of the

Bouin's fluid they were stained in the following elastic solution for 45 minutes.

Elastic solution

2% Haematoxylin in 95% alcohol	30ml
2.5% Ferric chloride in 1% HCl	20ml
Verhoeff's iodine	10ml

The tissue sections were then differentiated in equal parts of 2.5% ferric chloride and 95% alcohol.

The tissue embedding, sectioning and staining procedures described in sections 2.3.5 - 2.3.7 were carried out by the technical staff of the Department of Pathology, University of Edinburgh.

Table 2.3 summarises the tissue preparation procedures carried out on each available lung of the 13 subjects used in the technique validation studies.

2.3.8 Calculation of Tissue Shrinkage and Compression Resulting from Embedding and Sectioning

For three subjects (10-12, Table 2.1) duplicate sets of 12 tissue blocks were taken, one set embedded in paraffin, the other in glycol methacrylate. The size of each tissue block was measured before embedding (1.91cm x 1.91cm [$^3/_4$" x $^3/_4$"]) and after embedding and sectioning. Reduction in the size of the samples was known to be due to shrinkage only in one plane and to shrinkage plus compression in the other (the plane of section). The reduction due

Table 2.3 Summary of the tissue preparation procedures carried out on each available lung of the 13 study subjects.

Subject Number	Lungs Available	PA* Injection	Tissue Fixation	Tissue Sampling	Tissue Embedding
1	L	YES	routine	random	paraffin
	R	-			
2	L	-	"	"	"
	R	YES			
3	L	YES	"	"	"
	R	-			
4	L	YES	"	"	"
	R	-			
5	L only	-	"	"	"
6	L only	-	"	"	"
7	L only	-	"	"	"
8	L	-	uninflated	"	"
	R	-	routine		
9	L	-	uninflated	stratified	GMA#
	R	-	routine	random	
10	L	-	constant P+	"	paraffin
	R	-	routine		plus GMA
11	L	-	constant P	"	paraffin
	R	-	routine		plus GMA
12	L only	-	routine	"	paraffin plus GMA
13	R only	-	routine	"	GMA

* PA = Pulmonary artery

\+ P = Pressure

\# GMA = Glycol methacrylate

to shrinkage only was calculated initially and using this data a value for reduction due to compression could then be obtained. Shrinkage and compression in paraffin and glycol methacrylate were thus compared.

Alec McLean from the Department of Pathology, University of Edinburgh took the measurements and calculated the shrinkage and compression factors.

2.3.9 Measuring Equipment

All measurements were recorded using the Graphic Digitising Systems 1 equipment supplied by Graphic Information Systems Limited, Blairgowrie, Perthshire. This equipment, illustrated in Figure 2.6, comprises a digitising board with an electronic cursor together with a micro-computer (32K memory) and printer. Two types of electronic cursor were supplied, one with a light emitting diode intended for use in conjunction with a light microscope to obtain measurements directly from histological sections, the other with a cross-hair for obtaining measurements from photographs etc. taped to the digitising board. A hard copy unit, not illustrated in Figure 2.6, was also obtained. Using this unit permanent records of any information appearing on the display screen could be obtained.

Measurements of pulmonary arteries were obtained directly from histological sections by means of a 'camera lucida' attachment to the light microscope (in this case a Nikon Model S-Ke BR), the attachment orientated to overhang the digitising board. This arrangement enabled the pin-point of light emitted from the cursor

Figure 2.6 Photograph of the 'digitiser' set-up showing the component parts.

1 - Microscope with 'camera lucida' attachment

2 - Electronic cursor

3 - Digitising board

4 - Micro-computer / V.D.U.

5 - Printer

on the digitising board to be superimposed upon the artery in the field of view. Before commencing measurement, the digitising board was calibrated to the light microscope; this was effected by assigning each of the four buttons on the electronic cursor to a specific lens objective on the microscope. Various parameters of each artery, determined by the program used, were measured by moving the pin-point of light across or along the specified parts of the artery whilst depressing the appropriate button on the cursor. All co-ordinates 'activated' on the digitising board during the measurement of each parameter were recorded and passed to the microcomputer which translated the information into distances between points, lengths or areas depending on the parameter.

Measurements were also obtained from photographs by taping them to the digitising board, specifying the magnification, and drawing round the various elements with the cursor fitted with a cross-hair in place of a light emitting diode.

All measurements were printed directly and/or stored on tape for analysis at the operator's convenience. The data tapes used were Scotch 3M, standard length (100,000 bytes) or extra long (250,000 bytes), obtained from Graphic Information Systems Limited.

2.3.10 Methods for Measuring Muscular Pulmonary Arteries

In this section the intention is to describe exactly how the various parameters of each artery were measured in addition to describing the two programs used.

Program 1 was designed for measurement of the following parameters of muscular pulmonary arteries which satisfied the criteria of being cut in good cross-section and having a well-defined internal elastic lamina round the major part (at least 7/8ths) of their wall. The parameters measured by the observer are marked with an asterisk, measurements for the remaining parameters are produced by the micro-computer:-

* the two external diameters at right angles (D1, D2)
* the four corresponding medial thicknesses (M1, M2, M3, M4)
  the average medial thickness (MT)
* circumference of lumen (LC)
* total length of internal elastic lamina (IEL)
* external elastic lamina (EEL)
  area of lumen (LA)
  intima (IA)
  media (MA) (Figure 2.7)

Measurement of external diameters and medial thicknesses was facilitated by the use of an eye-piece graticule with a cross-hair. Since few of the cross-sectionally cut arteries were completely circular the cross-hair was orientated so that it intersected each artery at the points where both the long and short axes were at their maximum. The two external diameters were measured at the points where the cross-hair intersected the external elastic lamina, specifically its mid-point, and the four medial thicknesses where it intersected the external and internal elastic laminae, again specifically their mid-points. Use of the cross-hair eliminated the

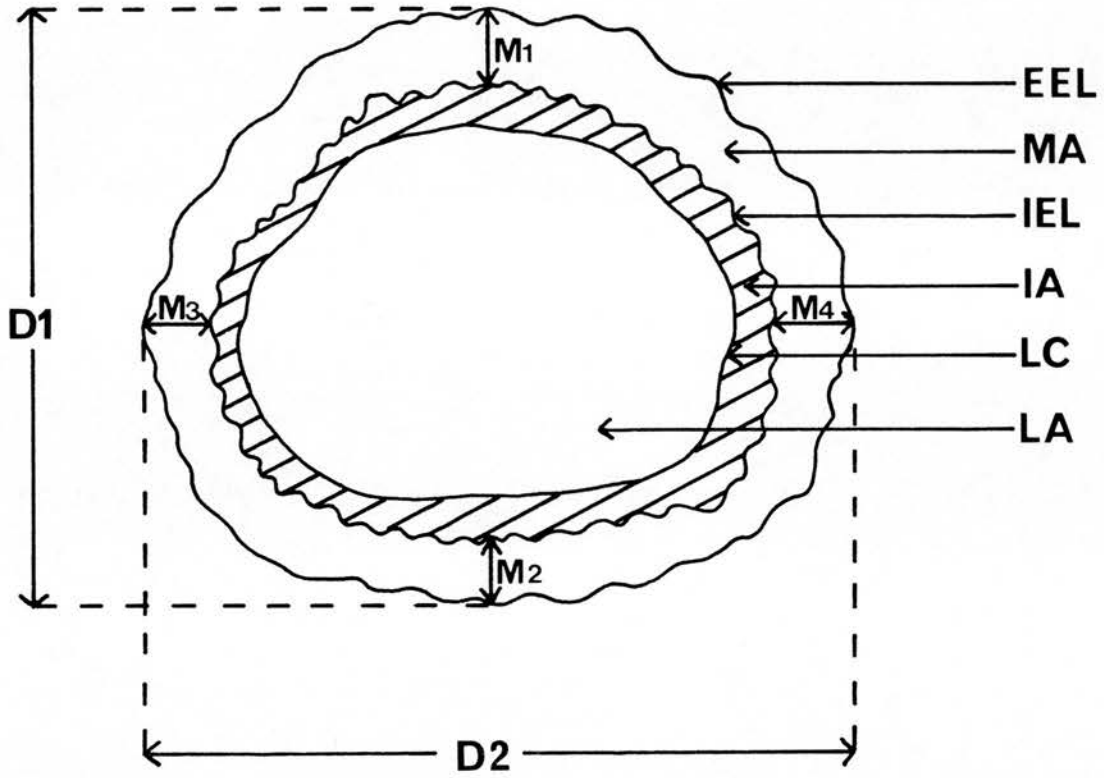


Figure 2.7 Diagrammatic representation of a muscular pulmonary artery indicating the structural components and measurements obtained using Program 1.
See text for explanation of abbreviations.

need for a subjective decision on where to take these measurements resulting in better reproducibility of the measurements.

Following measurement of external diameters and medial thicknesses the boundary between the lumen and intima was traced (lumen circumference) and the total lengths of the internal and external elastic laminae obtained by tracing the crinkles in the laminae. During the latter two measurements the light emitting diode was placed so that it lay centrally on each lamina.

Intimal area was calculated by the micro-computer as the area enclosed by the internal elastic lamina less the area enclosed by the lumen circumference. Similarly medial area was calculated as the area enclosed by the external elastic lamina less the area enclosed by the internal elastic lamina.

As already mentioned, to be considered measurable using the above technique an artery had to be cut in cross-section and have a well-defined internal elastic lamina. The latter was of prime importance as length of internal elastic lamina was to be used as an indicator of artery size. If its outline could not be seen clearly round at least $^{7}/_{8}$ths of any arterial wall, then that artery would not be considered measurable using the described technique. The external elastic lamina did not have to satisfy such stringent criteria as its length was considered a non-essential measurement, accurate delineation of the media being more important. Arteries satisfying the above criteria and measured using Program 1 were termed 'digitisable'.

Program 2 was designed to obtain more limited information for all muscular pulmonary arteries, both those considered 'digitisable' and the remainder. Each artery was assigned to a specific class depending on whether it was considered 'digitisable' (i.e. measurable using Program 1) or not, and also according to angle of cut, good cross-section or otherwise. For an artery cut in good cross-section the measurements made were as follows (Figure 2.8a):-

the two external diameters at right angles (D1A, D2A)
the two internal diameters at right angles (D1B, D2B)
the two lumen diameters at right angles (D1C, D2C)

For arteries not cut in good cross-section measurements were made on only the shorter axis of the artery at the point where it was widest in the plane perpendicular to its long axis (Figure 2.8b). Measurement of the lumen, internal and external diameters was again facilitated by the use of an eye-piece graticule with a cross-hair, the measurements being taken at the points where the cross-hair intersected the lumen - intima interface, the internal elastic lamina and the external elastic lamina respectively.

Medial and intimal thicknesses were calculated by subtraction, e.g. D1A - D1B, and D1B - D1C respectively; this data and the raw data on lumen, internal and external diameters were stored on tape.

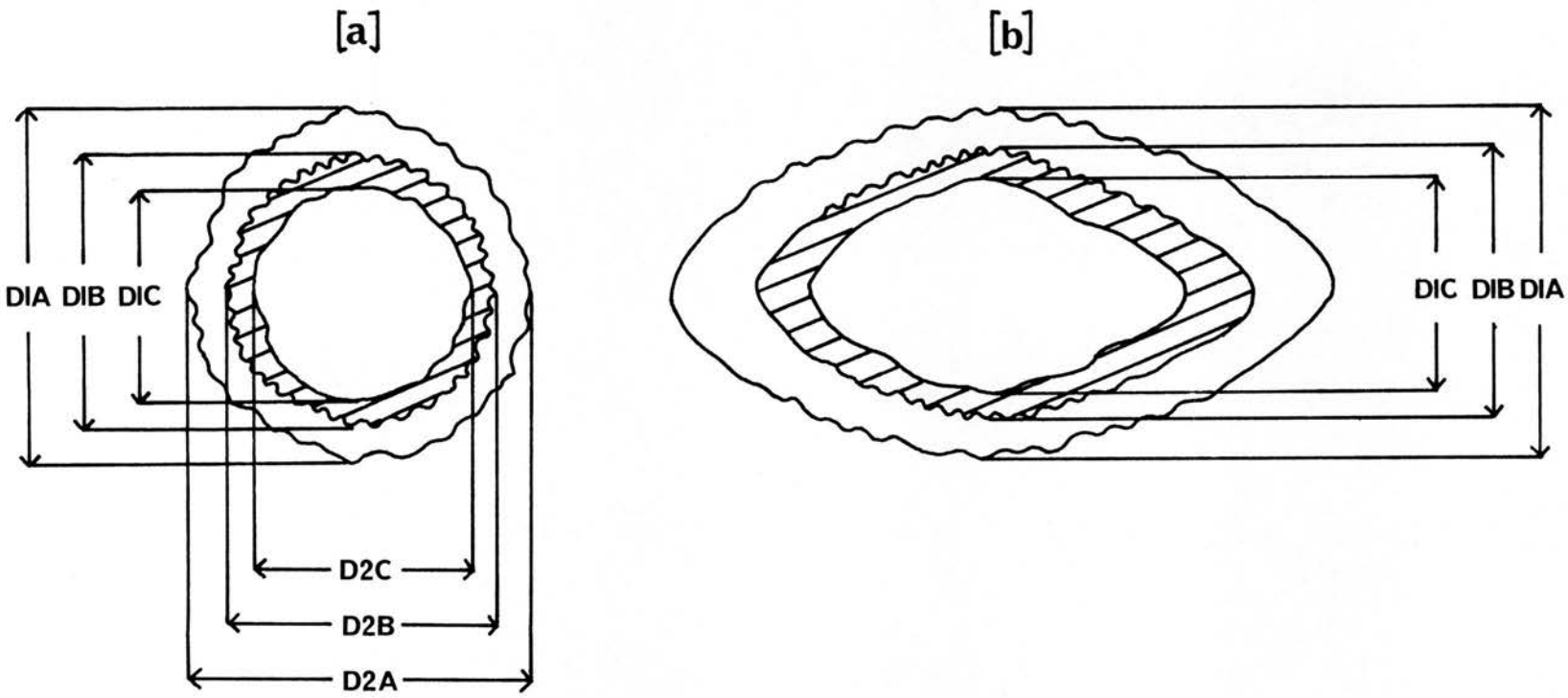


Figure 2.8 Diagrammatic representation of muscular pulmonary arteries cut in cross-section (a) or otherwise (b) and the measurements made using Program 2.

2.3.11 Method for Estimating Medial and Intimal Areas of Muscular Pulmonary Arteries

Efforts to maximise the number of arteries measured (see section 2.2 Aim 7) required a method which would provide estimates of medial and intimal area for those muscular pulmonary arteries which were cut in cross-section but which had an ill-defined internal elastic lamina. This was achieved by adapting the 'standard' measuring technique used in association with Program 1 (described in section 2.3.10). Instead of tracing the crinkles in the internal and external elastic laminae to obtain their total lengths (Figure 2.9a) the crinkles were ignored and the boundaries of the intimal and medial components delineated (Figure 2.9b). This technique was termed the 'abridged' technique to distinguish it from the 'standard' technique described in section 2.3.10. In addition to providing estimated values for medial and intimal area the 'abridged' technique produced values for the lengths of the boundary between intima and media, and media and adventitia.

2.3.12 By-eye Method for Grading the Degree of Constriction/ Collapse in Muscular Pulmonary Arteries

Assessment of the degree of constriction/collapse in an artery was based on the degree of 'crinkling' in the internal elastic lamina. It was graded on a scale of 0 through to 4 indicating none through to intense vasoconstriction. Typical examples of grades 1, 2, 3 and 4 are shown in Figure 2.10 a-d. Grade 0 is not illustrated. As one might imagine it represents an internal elastic

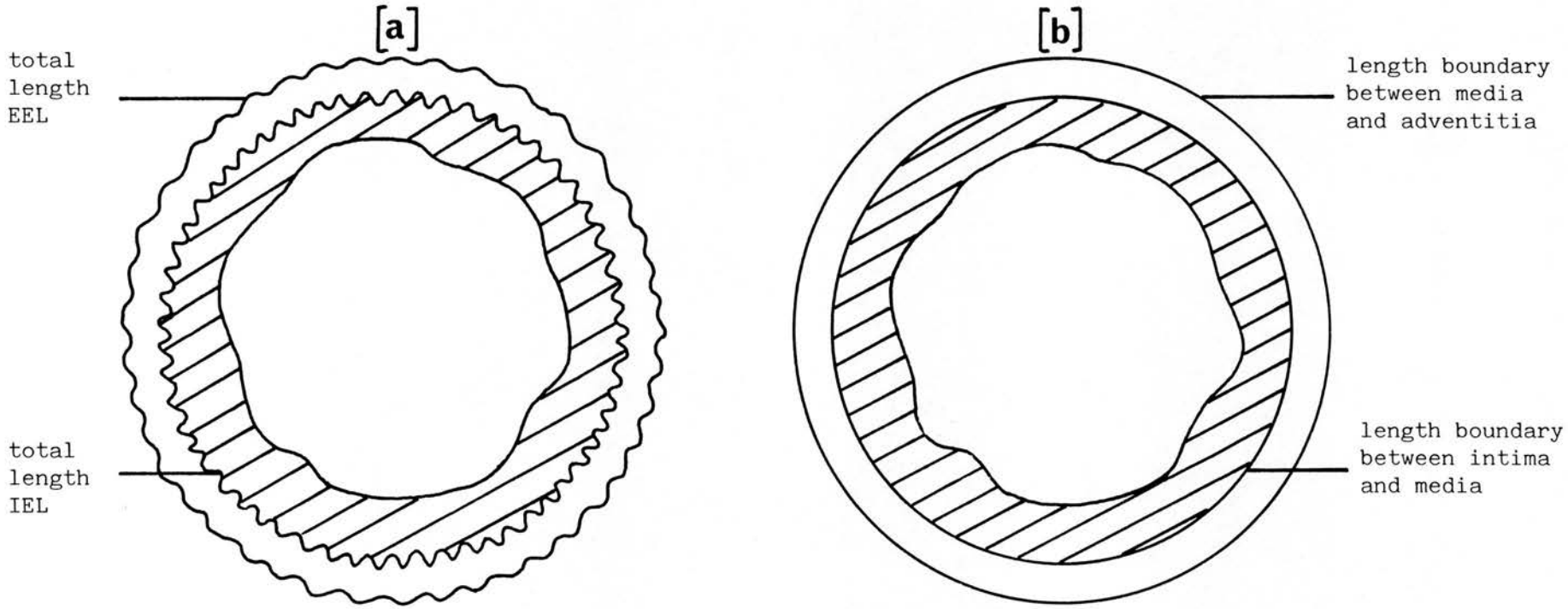
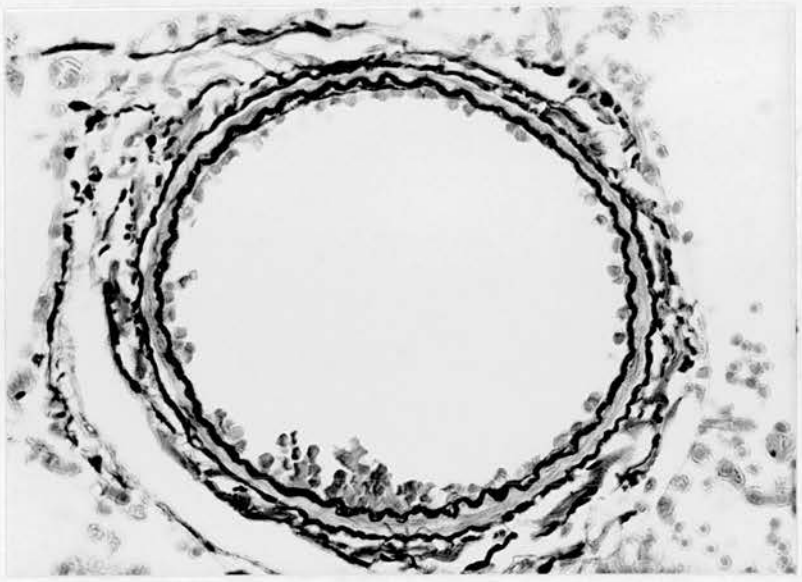
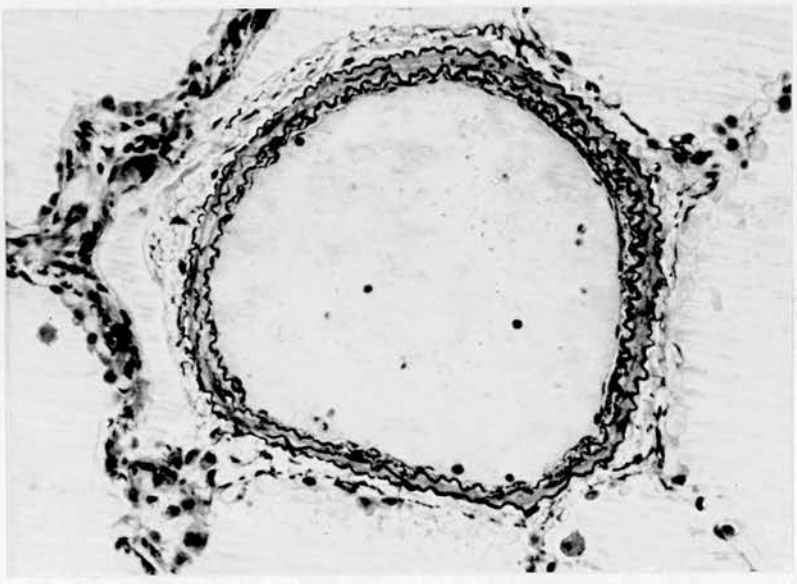


Figure 2.9 Diagrammatic representation of a muscular pulmonary artery indicating some of the measurements obtained using the standard (a) and abridged (b) technique associated with Program 1.



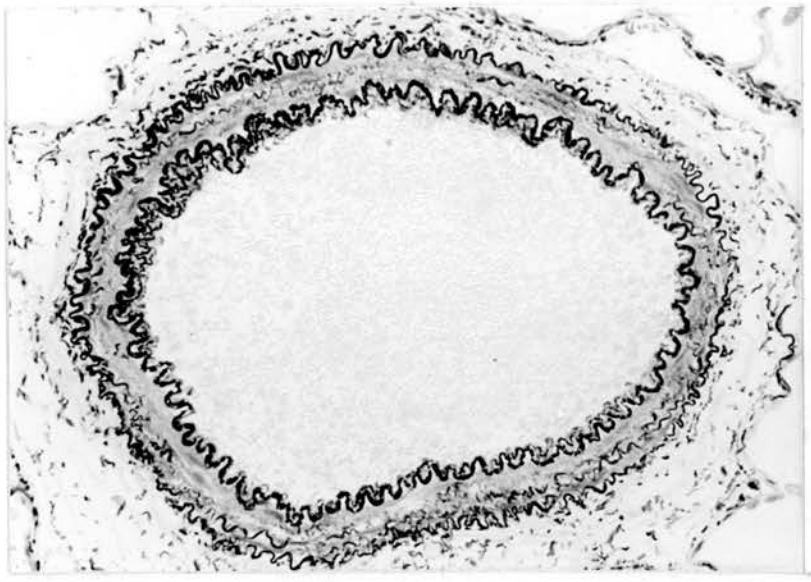
(a) x 400
Elastic Stain



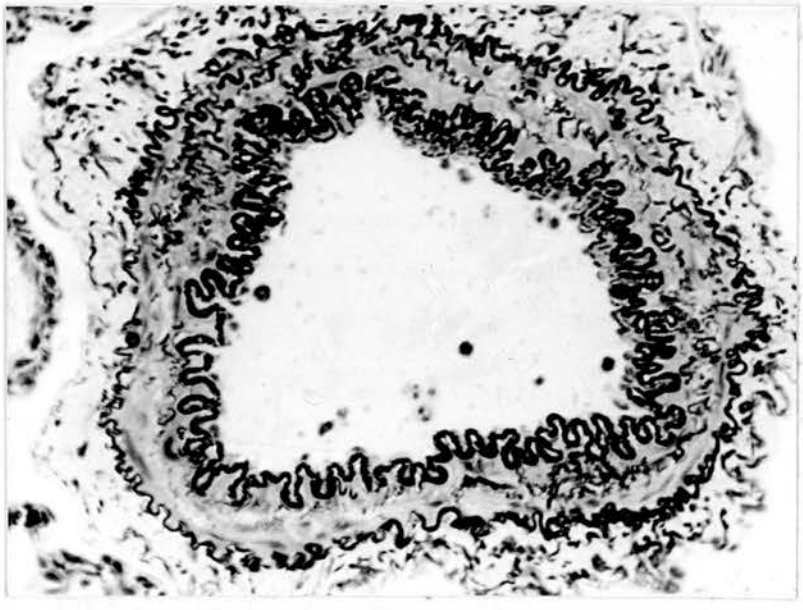
(b) x 250
Elastic Stain

(c) and (d) on next page

Figure 2.10 a - d Typical examples of grades 1, 2, 3 and 4 constriction/collapse in muscular pulmonary arteries.



(c) x 200
Elastic Stain



(d) x 375
Elastic Stain

Figure 2.10 continued.

lamina which is without 'crinkles'; this is rarely seen in uninjected arteries.

2.3.13 Method for Determining the Degree of Constriction/Collapse in Different Segments of an Internal Elastic Lamina

This was effected using a general quantitation program designed for measuring, amongst other parameters, 'Trace Lengths'. Using an eye-piece graticule composed of eight radiating spokes an artery was orientated so that its internal elastic lamina was sub-divided into eight approximately equal segments (Figure 2.11). Initially the total length of each segment of the internal elastic lamina was obtained by tracing the crinkles. These trace lengths were designated AB (1), BC (1), CD (1) etc. (Figure 2.11). A further set of measurements was then made, this time ignoring the crinkles in the internal elastic lamina and instead delineating the boundary between the intima and media. These trace lengths were designated AB (2), BC (2), CD (2) etc. (Figure 2.11). Segmental 'crinkle factors' were calculated by dividing AB (1) by AB (2) etc.

2.3.14 Programs Used for Data Analysis

The 'Simple Regressions' program was used for determining the relationship (if any) between any two variables. Various functions, linear and non-linear, are fitted to the data and the program selects the one giving the best fit. The functions are:-

$$y = Ax$$

$$y = A + Bx$$

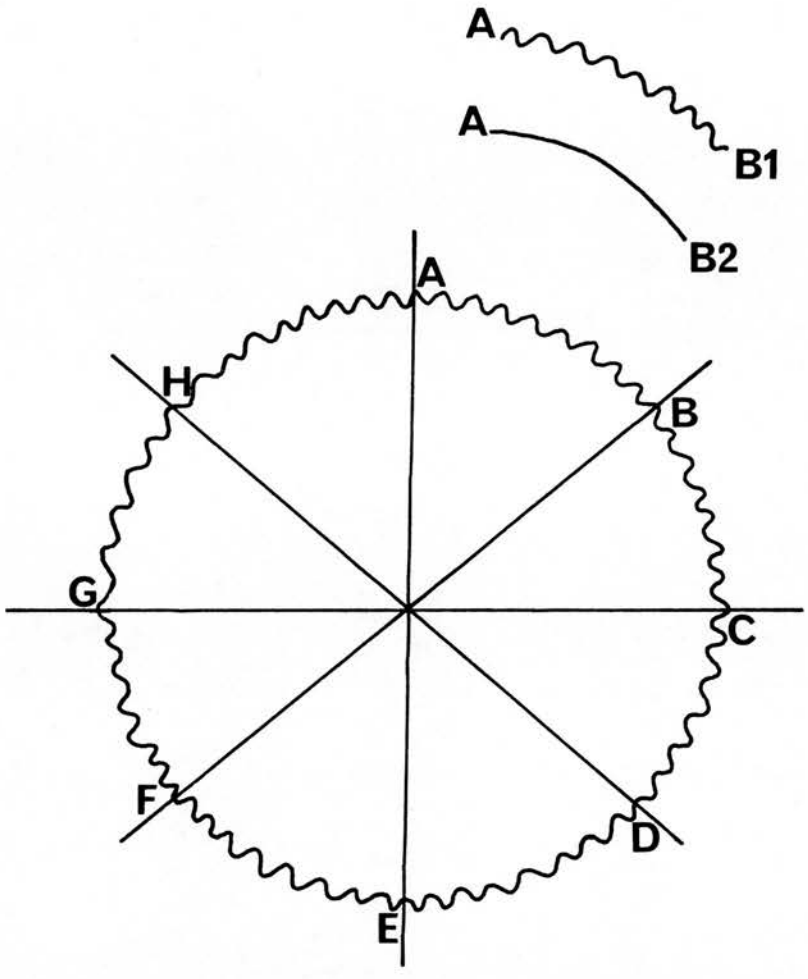


Figure 2.11 Diagrammatic representation of an internal elastic lamina indicating the measurements made using the 'Trace Lengths' program.

$$y = Ae^{Bx}$$

$$y = \frac{1}{A + Bx}$$

$$y = A + \frac{B}{x}$$

$$y = A + B.\log_e x$$

$$y = Ax^B$$

$$y = \frac{x}{A + Bx}$$

The '% wall thickness' program was used for calculating the medial and intimal thicknesses of arteries measured using Program 2 (see section 2.3.10) as a percentage of their external diameter.

The 'Intima Index' program was designed to enable the intimal area (IA) of arteries measured using Program 1 (see section 2.3.10) to be expressed as a ratio of the area enclosed by the internal elastic lamina (IEL) in its theoretically unwrinkled state (Figure 2.12).

$$\text{Intima Index} = \frac{\text{Area of intima}}{\text{Area enclosed by theoretically unwrinkled IEL}}$$

$$= \frac{IA}{(\text{length IEL}^2/4\pi)}$$

Values for Intima Index range from >0 to ⩽1. A value of 1 indicates total occlusion (actual and theoretical) of the lumen by intimal change.

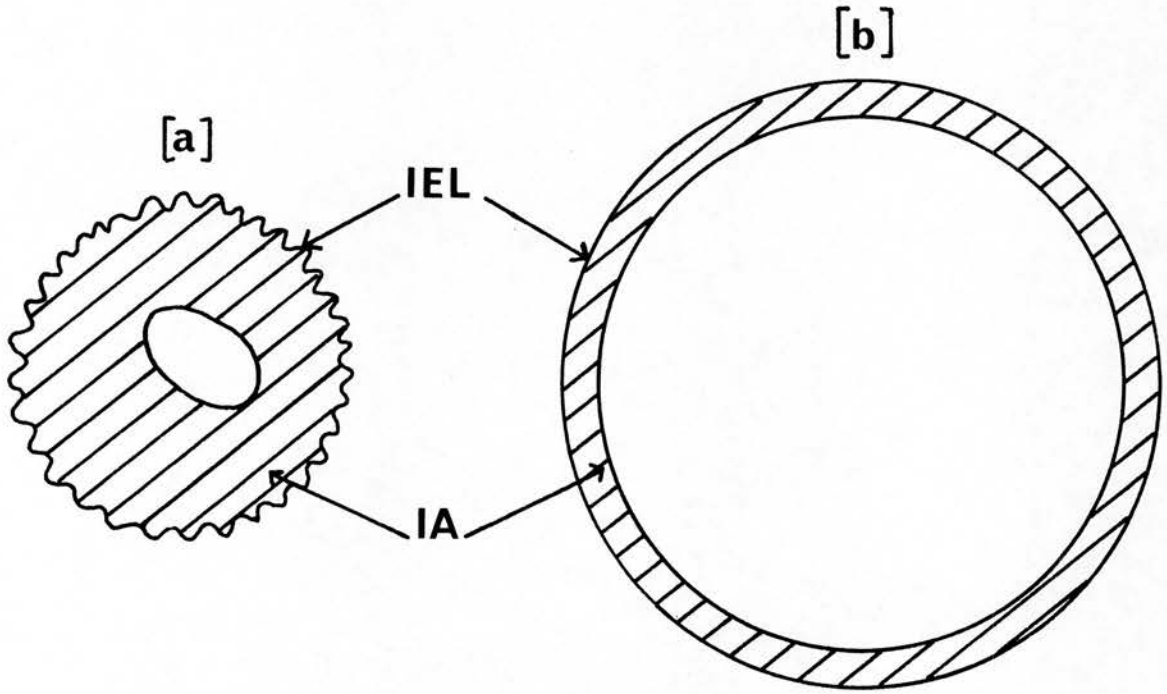


Figure 2.12 Diagrammatic representation of part of a muscular pulmonary artery showing the internal elastic lamina and intima in the measured (a) and theoretically unwrinkled (b) state.

2.3.15 Language and Source of Programs

All six programs were written in BASIC. Program 1 was written and supplied by Graphic Information Systems Limited following discussion with the author and Dr David Lamb from the Department of Pathology, University of Edinburgh. Also supplied by Graphic Information Systems Limited was a general quantitation program with a capacity for measuring 'Trace Lengths' (as described in section 2.3.13) together with a range of statistical programs including the 'Simple Regressions' program described in the previous section.

Program 2 (described in section 2.3.10) and the '% wall thickness' and 'Intima Index' programs described in the previous section were written by Mr Andrew Douglas of the Pathology Branch, Institute of Occupational Medicine in consultation with the author.

2.3.16 Statistical Analysis

For comparison of mean values the Student's t-test as described in Statistical Methods in Biology (Bailey, 1959) was used. The method used for paired t-tests was also as described in this book.

Comparison of two regression lines was done according to the method given by Wetherill (1972).

2.3.17 Assessment of Observer Error in the Measurement of Arteries of Known Dimensions

A model of a muscular pulmonary artery was made by drawing circles of radius 28mm, 42mm and 56mm on a piece of paper; these

circles represented the lumen - intima interface, the internal elastic lamina and the external elastic lamina respectively. The paper was taped to the digitising board and the 'artery' measured 10 times in succession using Program 1 standard procedure. Expected values for the measured parameters were calculated using the values for the three radii. Measured values were compared with the expected values and the maximum variation (+ and -) round the expected value expressed as a percentage of the expected value.

2.3.18 Assessment of the Effect of Constriction/Collapse on the Measurement of 'Internal Elastic Laminae' of Known Length

Models of 'internal elastic laminae' showing varying degrees of constriction/collapse were made from five pieces of strong thread of equal length, attached to paper sprayed with adhesive. Scale markings were drawn on each piece of paper. The five models were photographed and prints made at three different magnifications. Tracings of those of the lowest magnification for models 2-5 are illustrated in Figure 2.13 a-d. Model 1 is not illustrated; it was constructed as a perfect circle.

The photographs of the models were taped to the digitising board, and following calibration against the scale markings, the lengths of the 'internal elastic laminae' were measured using the 'Trace Lengths' program described in section 2.3.13.

In this section and in the previous section, the electronic cursor used was that with the cross-hair.

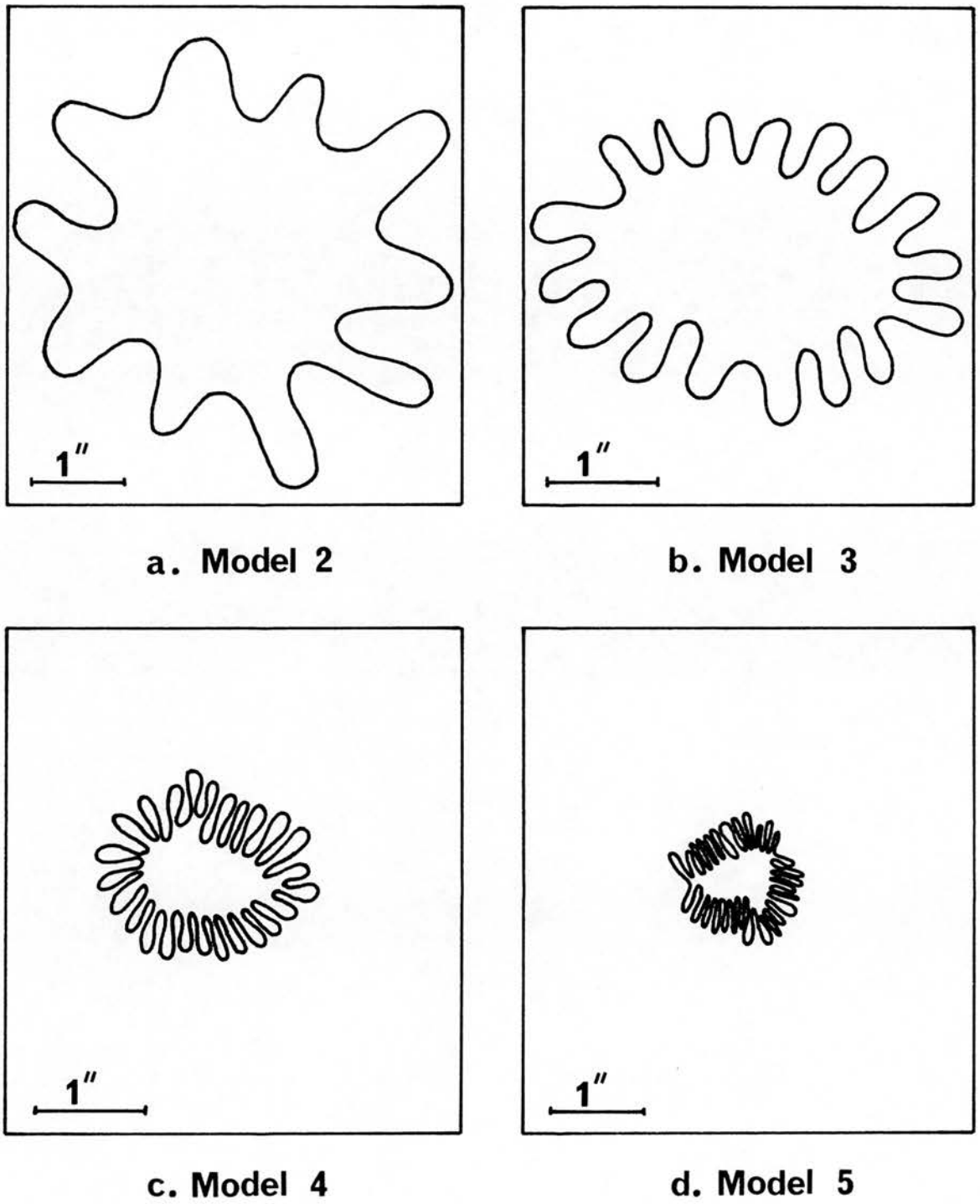


Figure 2.13 a - d Tracings of the models of 'internal elastic laminae' of equal length but showing different degrees of constriction/collapse.

2.3.19 Reproducibility of Measurements of Muscular Pulmonary Arteries

Fifteen muscular pulmonary arteries were selected from the histological sections of five subjects (1, 4, 5, 6 and 7, Table 2.1) whose pulmonary vasculature ranged from 'normal' (subject 5) to that associated with chronic obstructive lung disease (subject 1), rheumatic heart disease (subject 4), atrial septal defect (subject 6) and pulmonary sequestration (subject 7). These arteries were chosen for several reasons. They covered a wide range of abnormality, some possessed features which might affect repeatability of measurements, e.g. very thin media or extremely crinkled elastic laminae, and some were borderline in terms of being considered 'digitisable' (measurable using Program 1 standard procedure). Both injected and uninjected arteries were included. The features possessed by the 15 arteries are commonly seen in pulmonary arteries in both the normal and diseased states.

Each artery was digitised three times in succession using Program 1 standard procedure, and a mean value calculated for each parameter measured. All 15 arteries were then digitised on a further three occasions, each separated by a minimum period of six weeks. A sub-group of 10 arteries was additionally digitised at different magnifications.

Following analysis of the results obtained in this particular section and in the section on assessment of the effect of constriction/collapse on the measurement of 'internal elastic laminae' of known length (2.3.18) the described reproducibility

tests were repeated. In this second series of tests the point of light emitted from the diode on the electronic cursor was reduced in size so that it became a mere pin-point; this was done by blacking out the periphery of the light emitting diode using a felt-tip pen.

It had been considered sensible to assess reproducibility of measurements in injected as well as uninjected arteries since one of the aims of the chapter was to determine the effect of arterial distension on measurements of medial area and length of internal elastic lamina (see section 2.2 Aim 5 (i)). With regard to measurements of intimal area, and assessment of their reproducibility, there was no good reason for including injected arteries as the measuring technique was intended for use on uninjected arteries. The injected arteries were, therefore, ignored. This left only 11 arteries which were suitable for assessment of reproducibility of intimal area measurements. At this point it was decided to add in a further nine muscular pulmonary arteries; these were selected from subject 13 (Table 2.1). The inclusion of these nine extra arteries ensured that the 20 selected arteries covered the full size range of muscular pulmonary arteries, showed a wide variation in intimal abnormality in terms of type and extent, and also a wide variation in the degree of constriction/collapse present. As before, each artery was digitised three times in succession using Program 1 to obtain a base-line (mean) value for the parameter intimal area in particular, and once on a further three occasions, each separated by a minimum period of six weeks. All nine arteries were additionally digitised at different magnifications. Throughout, the measurements were done using the 'reduced' light emitting diode on the cursor.

2.3.20 Reproducibility of Selection of Arteries Considered 'Digitisable'

As described in section 2.3.10 a muscular pulmonary artery was considered 'digitisable' (measurable using Program 1 standard procedure) if it was cut in good cross-section and showed a well-defined internal elastic lamina round the major part (at least $^{7}/_{8}$ths) of its wall. To test the stringency of these criteria histological sections from four subjects were scanned on three occasions, each separated by a minimum period of four weeks, and the microscope stage co-ordinates (Vernier scale readings) of each artery considered 'digitisable' were recorded. The four subjects were those for whom injected and uninjected material was available (subjects 1-4, Table 2.1). These subjects were chosen because their pulmonary vasculature showed a wide range of abnormality and it was considered important to determine whether severity of disease affected selection of arteries considered 'digitisable'.

2.3.21 Comparison of 'Digitisable' Muscular Pulmonary Arteries with the Total Population

Histological sections from the four subjects used in the previous section were scanned to avoid including any field more than once, and all muscular pulmonary arteries identified. Measurements of each artery were made according to Program 2 as described in section 2.3.10.

2.4 RESULTS

The results sections are ordered as follows. Sections involving reproducibility testing come first (sections 2.4.1 - 2.4.5) followed by those involving assessment of the effect of different tissue preparation methods on measurements of the media and size of muscular pulmonary arteries (sections 2.4.6 - 2.4.11). Those involving assessment of the intimal component come next (sections 2.4.12 - 2.4.14) and lastly come the sections concerned with maximising the data obtainable from muscular pulmonary arteries (sections 2.4.15 - 2.4.19).

Much of the work reported has already been written up and accepted for publication. Archives of Pathology and Laboratory Medicine have accepted two papers, one based on sections 2.4.3 - 2.4.5, the other based on sections 2.4.6 - 2.4.11.

2.4.1 Assessment of Observer Error in the Measurement of 'Arteries' of Known Dimensions

A model of a muscular pulmonary artery of known dimensions was measured 10 times in succession using Program 1 as described in section 2.3.17. Table 2.4 illustrates the actual values for the nine parameters and also the mean values calculated from the 10 repeat measurements. The maximum deviation (+ or -) from the actual value, expressed as a percentage of the actual value, is also given for each parameter. These results indicate that there is some element of operator error even in the measurement of very simply constructed models of pulmonary arteries, using the digitiser. On

Table 2.4 Operator error in the measurement of a model of a muscular pulmonary artery of known dimensions.

	Artery Parameters								
	D1	D2	MT	LA	LC	IA	IEL	MA	EEL
Actual Value*	112.0	112.0	14.0	2464.0	176.0	3080.0	264.0	4312.0	352.0
Mean of 10 measurements	111.6	111.8	14.1	2432.2	175.0	3035.2	262.7	4321.9	351.5
Maximum % deviation from actual value	0.5	0.3	0.7	1.9	1.9	1.9	0.7	0.7	0.3

* expressed in mm or mm^2

see text (p 82) for explanation of parameter abbreviations

any given occasion, however, one would expect these errors in measurement to be certainly less than 2% and in most instances less than 1%.

2.4.2 Assessment of the Effect of Constriction/Collapse on the Measurement of 'Internal Elastic Laminae' of Known Length

Throughout this study artery size was to be defined in terms of the total length of the internal elastic lamina. Accordingly, this parameter was considered the most critical and in many arteries the most difficult to measure depending on the degree of collapse/constriction present. It was, therefore, thought essential to determine how accurate the measurements obtained using the digitiser were.

Photographs of models of 'internal elastic laminae' showing varying degrees of constriction/collapse (illustrated in Figure 2.13) were measured using the 'Trace Lengths' program as described in section 2.3.18. The 'internal elastic laminae' were of equal length, 24.20 inches. Table 2.5 shows the measured lengths of these 'laminae' and the extent, expressed as a percentage, by which they deviated from the actual value. Increasing degree of constriction/collapse in the 'lamina' was associated with an increasing error in the measurement of this parameter at low magnification which was 9% for the most collapsed/constricted lamina, number 5. For 'laminae' numbers 1 and 2 the errors of 1.7% were within the expected range in view of the results obtained in the previous section. The errors of 2.9% and 3.1% for 'laminae' numbers 3 and 4 respectively, were

Table 2.5 The effect of constriction/collapse on measurements of 'internal elastic laminae' of known length 'viewed' at different magnifications. Measured values quoted; % deviation from actual value of 24·20 given in brackets.

Model Number+	Magnification* Low	Intermediate	High
1	24.60# (1.7)	24.53 (1.7)	24.48 (1.2)
2	23.80 (1.7)	24.26 (0.2)	24.11 (0.4)
3	23.49 (2.9)	23.40 (3.3)	23.35 (3.5)
4	23.45 (3.1)	23.80 (1.7)	23.81 (1.6)
5	22.02 (9.0)	23.61 (2.4)	23.85 (1.4)

* Magnifications were not standard for all models.

The ranges were:

Low	0.6 - 0.7
Intermediate	0.8 - 1.4
High	1.5 - 2.0

+ Moving from model 1 through to model 5 there is an increasing degree of constriction/collapse in the'elastic laminae'.

expressed in inches

higher, but not unduly higher than expected. It was only with model 5 that the errors were considered less than acceptable. In general, increasing magnification helped to reduce these errors in measurement, especially so for model 5 where they were reduced to the expected value.

These findings have important implications regarding the use of the internal elastic lamina as an indicator of artery size. They indicate that the measurements of this parameter produced using the digitiser are very accurate providing the measurement is done at a magnification at which the crinkles in the lamina are distinct and easy to trace. This point is further pursued in sections 2.4.3 (iii) and (iv).

2.4.3 Reproducibility of Measurements of Muscular Pulmonary Arteries

(i) Short-term

For each of the 15 muscular pulmonary arteries, selected as detailed in section 2.3.19 and measured using Program 1, mean values of all parameters were calculated from those obtained at three consecutive digitisations. The parameter intimal area is not included in this section but is reported on later (section 2.4.12). By so doing it was possible to keep all sections relating to assessment of the intima together.

Actual values for each parameter/artery were considered unimportant and are not quoted. For each parameter/artery the maximum (+ or -) percentage deviation from the mean was calculated

to give some indication of the spread of the values. From Table 2.6 it can be seen that the values obtained for all 15 arteries on consecutive digitisations mostly lay within 2% of the mean value and a high proportion were within 1%. This was evident for all parameters with the notable exception of average medial thickness (MT) where deviations of up to 11.3% were observed.

(ii) Long-term

Using the mean values obtained from the initial three consecutive digitisations as a base-line, the long-term reproducibility of the measurements was investigated as described in section 2.3.19. On each of three further digitisations the percentage deviation from the base-line value was calculated for each parameter/artery. Table 2.7 shows the maximum percentage deviation observed over the three independent digitising sessions for each parameter/artery, together with the notable features of each artery, if any. Of the 120 values illustrated 93 show a maximum deviation of less than 5% from the base-line value. Poor reproducibility of the medial thickness (MT) parameter, which was not unexpected, accounted for 10 of the remaining 27 values. In general, poorer reproducibility (greater than 5% deviation) of a parameter other than MT in any artery was linked to its structure, e.g. very thin media or very crinkled internal elastic lamina, or its size.

(iii) Effect of magnification

The effect of magnification on the measurements obtained for each parameter was investigated in a sub-group of 10 muscular pulmonary arteries which fulfilled the requirement of being

Table 2.6 Number of arteries in which the measurements obtained at three consecutive digitisations deviated by more than one or two percent from the mean value.

% deviation from mean value	Artery Parameters							
	D1	D2	MT	LA	LC	IEL	MA	EEL
> 1%	1	2	14	1	2	5	6	4
> 2%	0	0	12	0	0	3	2	1

see text (p 82) for explanation of parameter abbreviations

Table 2.7 The long-term reproducibility of the measurements of 15 arteries expressed as the maximum (+ or -) percentage deviation from the base-line value. Notable features of each artery, if any, are given.

Artery Number	Artery Parameters								
	D1	D2	MT	LA	LC	IEL	MA	EEL	Notable Features
1*	3.1	1.9	12.9	3.4	1.7	1.7	7.4	1.6	very thin media
2*	0.9	1.1	4.0	1.4	0.9	1.2	1.6	1.8	-
3*	2.4	2.5	4.0	2.4	1.7	1.8	7.0	0.7	very thin media
4*	4.2	0.4	5.5	2.5	1.3	0.9	2.4	0.8	very thin media
5	1.3	0.5	3.9	0.9	0.2	1.7	7.3	0.6	-
6	4.1	2.2	8.3	5.8	2.7	2.9	4.2	2.4	-
7	0.9	1.3	5.1	0.3	0.4	0.6	2.1	0.4	-
8	5.9	3.7	5.5	5.1	3.4	1.8	3.9	3.9	partly ill-defined/ crinkled elastic laminae
9	4.5	2.8	8.8	2.4	2.5	3.6	3.3	3.5	partly ill-defined/ crinkled elastic laminae
10	4.6	2.3	1.4	5.4	1.8	3.8	11.7	1.6	-
11	0.9	1.0	7.2	2.8	1.0	0.8	2.1	1.0	-
12	0.9	1.7	1.3	1.1	1.6	5.6	1.6	4.4	very crinkled elastic laminae
13	1.0	1.2	5.2	1.3	0.4	7.2	1.1	1.3	very crinkled elastic laminae
14	4.6	2.5	9.2	7.7	5.2	13.0	3.4	13.8	very small artery
15	1.9	0.4	14.0	2.1	1.6	6.4	3.4	6.6	partly ill-defined elastic laminae

* injected artery

see text (p 82) for explanation of parameter abbreviations

'digitisable' at a minimum of three out of four lens objective magnifications: x4, x10, x20 and x40. Each artery was measured once at the appropriate magnifications using Program 1. The data are expressed as ratios:-

$$\frac{\text{measurement at specified magnification}}{\text{measurement at lowest magnification}}$$

and are arranged in order of increasing magnification (Table 2.8). Thus, a ratio of 1 throughout for any parameter would indicate that that particular measurement was unaffected by magnification.

To start with a general comment, the speed of obtaining the measurements decreased with increasing magnification, as one might expect. With regard to the measurements themselves the results illustrated in Table 2.8 suggest that the diameter (D1, D2) and area (in particular LA) measurements were generally less affected by magnification than the length (LC and particularly IEL and EEL) measurements. This was most obvious in arteries showing marked crenation of the elastic laminae (numbers 8, 9 and 13). The results for the parameter medial area (MA) were rather mixed. Six of the arteries (numbers 1, 6, 7, 10, 14 and 15) showed relatively low ratios indicating that the area had been considerably over-estimated by measurement at the lowest magnification. However, the ratios were fairly constant throughout suggesting that the problem was present only at the lowest magnification. The error in measurement of medial area at low magnifications for these arteries is probably explained by the fact that three arteries were injected (numbers 1, 6 and 7) and therefore very thin-walled, the other three (numbers 10, 14 and 15) fairly small. Ratios for the medial thickness (MT)

Table 2.8 Data for the parameters of 10 arteries digitised at different lens objective magnifications, expressed as the ratio of measurement at specified magnification: measurement at lowest magnification (x4 for all arteries except number 1).

Artery Number	Specified Mag^{n}.	Artery Parameters D1	D2	MT*	LA	LC	IEL	MA	EEL
1	x20	0.99	0.99		1.01	1.01	0.99	0.74	0.99
	x40	0.99	1.01		1.07	1.03	1.03	0.69	1.00
6	x10	0.98	0.97		1.04	1.02	1.01	0.82	0.99
	x20	1.01	0.96		1.11	1.06	1.05	0.80	1.01
	x40	1.01	0.96		1.11	1.06	1.05	0.76	1.02
7	x10	0.90	1.03		1.03	1.02	1.01	0.85	1.04
	x20	0.97	1.00		1.08	1.05	1.06	0.87	1.06
	x40	0.96	1.01		1.08	1.05	1.05	0.84	1.06
8	x10	1.01	1.01		1.00	1.02	1.26	0.94	1.07
	x20	1.01	1.03		1.04	1.06	1.52	1.01	1.22
9	x10	0.98	0.98		1.05	1.03	1.16	0.94	1.13
	x20	1.00	0.99		1.11	1.10	1.37	0.98	1.33
10	x10	1.00	1.01		1.03	1.05	1.04	0.81	1.02
	x20	0.99	1.00		1.04	1.02	1.04	0.83	1.05
	x40	1.00	1.02		1.08	1.04	1.10	0.84	1.11
11	x10	0.98	0.98		1.00	1.02	1.03	0.98	1.01
	x20	1.00	0.99		1.05	1.06	1.07	1.03	1.04
13	x10	0.98	1.03		1.00	1.01	1.43	0.99	1.25
	x20	1.00	1.02		1.04	1.05	1.55	1.04	1.38
14	x10	0.94	0.91		1.11	1.01	1.08	0.56	1.01
	x20	0.88	0.92		1.18	1.09	1.34	0.54	1.18
	x40	0.89	0.92		1.19	1.10	1.29	0.51	1.22
15	x10	0.94	0.96		1.02	1.00	1.05	0.84	1.01
	x20	0.98	0.99		1.07	1.08	1.18	0.82	1.10
	x40	0.98	0.99		1.09	1.12	1.23	0.85	1.14

* Ratios not quoted, see text for explanation

See text (p 82) for explanation of parameter abbreviations

parameter are not quoted as they were considered grossly inaccurate at the lowest magnification.

(iv) Effect of reducing the size of the point of light emitted from the cursor

While carrying out the above reproducibility tests the electronic cursor used was the one with the light emitting diode since the measurements of the arteries were taken directly from the histological sections. It was intuitively felt throughout that the 'point' of light emitted was far too large; with some of the injected, and therefore thin-walled arteries, it was almost as thick as the wall itself. Because of this the errors in measurement of medial area at low magnifications for some arteries were hardly surprising, nor for that matter were the errors in the measurement of some elastic laminae. In an attempt to overcome this problem, the point of light was reduced in size to a mere pin-point by blacking out the periphery of the light emitting diode. All reproducibility tests described in sections (i) - (iii) were repeated. The results are illustrated in Tables 2.9, 2.10 and 2.11. A comparison of Table 2.6 with 2.9 and Table 2.7 with 2.10 revealed no consistent or significant differences in either the short or long-term reproducibility of measurements, with the exception of the MT parameter in which greater errors were incurred using the 'reduced' light emitting diode. This is probably explained by the relatively greater 'choice' available on where to take this measurement in situations where the cross-hair did not intersect an elastic lamina cleanly but rather was superimposed on it for some distance. Reducing the light emitted from the cursor to a mere pin-

Table 2.9 Number of arteries in which the measurements obtained* at three consecutive digitisations deviated by more than one or two percent from the mean value.

% deviation from mean value	Artery Parameters							
	D1	D2	MT	LA	LC	IEL	MA	EEL
>1%	2	0	14	0	1	6	4	4
>2%	0	0	14	0	0	2	1	3

* Obtained using the 'reduced' light emitting diode on the cursor

See text (p 82) for explanation of parameter abbreviations

Table 2.10 The long-term reproducibility of the measurements* of 15 arteries expressed as the maximum (+ or -) percentage deviation from the base-line value. Notable features of each artery, if any, are given.

Artery Number	Artery Parameters D1	D2	MT	LA	LC	IEL	MA	EEL	Notable Features
1+	0.9	1.4	10.0	2.9	1.1	1.1	8.3	1.1	very thin media
2+	1.3	1.2	9.8	0.7	0.2	0.8	2.1	2.4	-
3+	0.9	0.4	8.7	0.9	1.1	1.2	6.0	0.2	very thin media
4+	0.9	0.8	14.6	0.7	0.4	0.5	5.2	0.5	very thin media
5	1.1	1.1	16.3	0.3	1.9	1.6	5.5	2.6	-
6	0.2	1.3	8.3	1.3	1.1	1.7	0.7	0.9	-
7	1.3	4.4	9.3	0.9	1.5	3.7	1.9	1.6	-
8	0.9	1.4	21.1	1.4	0.5	4.0	3.0	5.3	partly ill-defined/ crinkled elastic laminae
9	2.3	1.5	2.2	2.6	2.2	9.7	0.9	2.1	partly ill-defined/ crinkled elastic laminae
10	0.8	1.4	8.5	1.5	1.3	4.9	1.3	1.9	-
11	3.4	1.0	10.7	0.8	1.5	2.5	2.2	1.3	-
12	0.6	0.4	5.4	0.6	1.2	3.7	0.3	3.7	very crinkled elastic laminae
13	1.6	1.8	4.8	1.8	3.1	5.3	2.2	5.2	very crinkled elastic laminae
14	3.7	2.7	21.9	8.5	6.1	6.3	10.0	8.3	very small artery
15	2.8	1.6	17.7	1.5	0.7	1.6	2.7	7.1	partly ill-defined elastic laminae

* Obtained using the 'reduced' light emitting diode on the cursor

+ injected artery

See text (p 82) for explanation of parameter abbreviations

Table 2.11 Data for the parameters of 10 arteries digitised* at different lens objective magnifications, expressed as the ratio of measurement at specified magnification: measurement at lowest magnification (x4 for all arteries except number 1).

Artery Number	Specified Magn.	Artery Parameters D1	D2	MT+	LA	LC	IEL	MA	EEL
1	x20	1.01	1.01	0.33	1.03	1.02	1.01	0.97	0.99
	x40	1.04	1.03	0.38	1.08	1.04	1.04	1.01	1.03
6	x10	1.03	0.99	0.40	1.09	1.05	1.03	0.96	1.07
	x20	1.02	1.02	0.25	1.13	1.06	1.05	0.96	1.11
	x40	1.03	1.03	0.26	1.15	1.08	1.06	0.98	1.10
7	x10	0.94	1.00	0.33	0.98	0.99	1.00	1.01	0.98
	x20	0.96	0.98	0.24	1.00	1.01	1.03	1.02	0.99
	x40	0.97	1.01	0.26	1.03	1.02	1.05	1.03	1.00
8	x10	0.97	1.00	0.71	1.01	1.06	1.20	0.95	1.04
	x20	0.97	1.02	0.66	1.05	1.09	1.24	1.00	1.11
9	x10	0.99	1.01	0.51	0.99	0.99	1.11	0.98	1.17
	x20	1.00	1.01	0.53	1.00	1.01	1.22	1.02	1.23
10	x10	0.97	0.98	0.38	0.99	1.04	0.99	0.96	1.02
	x20	0.99	1.00	0.38	1.03	1.06	1.04	0.97	1.05
	x40	1.00	1.02	0.38	1.06	1.08	1.07	1.03	1.09
11	x10	0.99	0.99	0.86	0.97	1.04	1.05	0.99	1.01
	x20	1.01	1.01	0.87	1.01	1.04	1.06	1.02	1.06
13	x10	0.99	0.99	0.97	0.97	1.00	1.18	0.98	1.14
	x20	1.02	1.02	0.99	1.00	1.04	1.30	1.02	1.20
14	x10	1.01	0.97	0.49	1.01	0.99	1.06	0.87	1.16
	x20	1.01	0.96	0.24	1.05	1.01	1.14	0.81	1.23
	x40	1.00	0.96	0.25	1.05	1.01	1.19	0.77	1.24
15	x10	1.02	0.96	0.40	1.02	1.01	1.10	0.85	1.02
	x20	1.04	0.98	0.24	1.05	1.07	1.15	0.88	1.11
	x40	1.04	1.00	0.26	1.06	1.10	1.16	0.89	1.12

* Digitised using the 'reduced' light emitting diode on the cursor

+ The low MT ratios for most arteries are explained by gross inaccuracies in the measurement of this parameter at low magnification

See text (p 82) for explanation of parameter abbreviations

point did, however, have the desired effect in reducing the errors associated with the measurement of certain parameters at low magnification (Table 2.8 compared with 2.11). For instance, the medial area ratios of arteries 1, 6, 7 and 10 now approximated 1 throughout, and while there were still some problems with some of the smaller arteries, such as 14 and 15, the ratios were nearer 1 than they had been. Improvements were also noted in the measurement of extremely crinkled elastic laminae as in arteries 8, 9 and 13. These improvements have important implications, particularly with respect to measurement of large muscular pulmonary arteries which, because of their size, have to be measured at lower magnifications.

The conclusions to be drawn from the reproducibility tests described in sections 2.4.1 - 2.4.3 are that reproducibility of measurements obtained using the digitiser is good. Magnification does affect the accuracy of measurements obtained for some parameters, notably the internal elastic lamina, so it is essential to digitise at a magnification at which the crinkles in the elastic lamina are clearly defined.

2.4.4 Reproducibility of Selection of Arteries Considered 'Digitisable'

To be considered 'digitisable' (measurable using Program 1 standard procedure) a muscular pulmonary artery had to be cut cross-sectionally and have a well-defined internal elastic lamina round at least $^{7}/_{8}$ths of its wall. It was considered sensible to determine how stringent these criteria were. Accordingly, the histological sections from four subjects (1-4, Table 2.1) were scanned on three

separate occasions and each muscular pulmonary artery considered 'digitisable' was identified by the microscope stage co-ordinates at which it lay in the centre of the field of view. Table 2.12 describes the results which show a high level of consistency in the selection of arteries considered 'digitisable' thus indicating that the criteria for 'digitisability' were adequately stringent. The results were additionally encouraging because the pulmonary arteries of the four selected subjects were quite diseased, especially subject 4, and it was, therefore, reassuring to find that the selection of arteries for measurement was not affected by severity of disease present.

2.4.5 Comparison of 'Digitisable' Arteries with the Total Muscular Pulmonary Artery Population

It was observed (Table 2.12) that the number of muscular pulmonary arteries considered 'digitisable' was very few in some subjects; the main reason for arteries not being considered 'digitisable' was that they were not cut in good cross-section. This gave rise to the question as to whether the 'digitisable' arteries could truly be considered representative of the total muscular pulmonary artery population in terms of the amount of medial muscle and intimal abnormality present in an artery of a given size. As measurements of 'undigitisable' arteries were of necessity limited to medial and intimal thicknesses and diameter the comparison of 'digitisable' arteries with the total population was based on these parameters.

Table 2.12 Number of muscular pulmonary arteries considered 'digitisable' on three separate screenings together with the number common to all three screenings.

Subject Number	Lung	1st screening	2nd screening	3rd screening	common
1	injected (12)	28	28	30	26
	uninjected (12)	31	30	33	26
2	injected (5)	38	38	37	32
	uninjected (11)	19	17	17	16
3	injected (11)	48	45	49	42
	uninjected (11)	11	13	13	10
4	injected (9)	14	15	15	14
	uninjected (10)	27	27	25	22

No. of histological sections given in brackets

Using the same four subjects as in the previous section measurements of all muscular pulmonary arteries were made using Program 2 as described in section 2.3.10. Since the majority of arteries were not cut cross-sectionally the short diameter was used as the indicator of size. The muscular pulmonary arteries were subdivided by external diameter (DIA) into six groups and the mean medial thickness (DIA - DIB) and the mean percent medial thickness $\left\{\frac{D1A - D1B}{D1A} \times \frac{100}{1}\right\}$ calculated for each size group for both 'digitisable' arteries and the total population. Similarly, the mean intimal thickness (D1B - D1C) and percent intimal thickness $\left\{\frac{D1B - D1C}{D1A} \times \frac{100}{1}\right\}$ were calculated. The results for all four subjects showed similar trends and only those of subject 1 are illustrated, the media in Table 2.13, and the intima in Table 2.14. The data presented in these tables indicate that the 'digitisable' arteries comprised a small porportion of the total population, none of the arteries being considered 'digitisable' in some size groups and very few in others. Overall, there was a tendency for a greater proportion of the larger arteries to be considered 'digitisable'.

In terms of mean percentage medial thickness values (Table 2.13) or mean percentage intimal thickness values (Table 2.14) there appeared to be no striking differences between 'digitisable' arteries and total population in any of the size groups. Statistical testing of the significance of the difference between these mean values was hampered in some size groups by an insufficient number of 'digitisable' arteries, and in others by an unbalanced distribution of arteries between the 'digitisable' and total population groups. In those size groups in which it was

Table 2.13 Mean percentage medial thickness values for the muscular pulmonary arteries, 'digitisable' and total population, of subject 1 sub-divided by external diameter.

Lung	Artery Population*	External Diameter : DIA											
		< 100μm		100-199μm		200-299μm		300-399μm		400-499μm		⩾ 500μm	
		n+	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)
injected	D	-		8	6.0(1.0)	7	5.1(1.7)	6	4.3(1.1)	3	5.0(17)	8	4.3(0.6)
	T	22	10.5(4.5)	124	6.4(1.6)	74	5.0(1.4)	37	4.7(1.2)	19	4.3(1.2)	25	4.3(1.1)
uninjected	D	2	14.7(1.9)	16	10.6(3.0)	9	11.1(2.2)	3	12.1(1.9)	2	9.6(0.6)	2	13.1(0.2)
	T	82	14.0(4.5)	154	11.2(3.0)	66	11.3(3.3)	21	11.5(3.1)	6	12.4(3.1)	12	13.8(3.3)

* D = 'digitisable' arteries, T = total population

+ n = number of arteries

Table 2.14 Mean percentage intimal thickness values for the muscular pulmonary arteries, 'digitisable' and total population, of subject 1 subdivided by external diameter.

Lung	Artery Population*	External Diameter : DIA											
		< 100μm		100-199μm		200-299μm		300-399μm		400-499μm		⩾ 500μm	
		n+	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)
injected	D	-		8	6.1(2.0)	7	3.6(0.8)	6	3.6(1.2)	3	1.7(1.1)	8	2.1(1.1)
	T	22	7.0(2.7)	124	5.6(2.2)	74	3.6(1.1)	37	3.2(1.1)	19	2.5(1.1)	25	1.9(0.9)
uninjected	D	2	13.0(8.3)	16	11.1(4.4)	9	8.6(3.2)	3	4.7(1.7)	2	5.7(0.7)	2	5.6(0.4)
	T	82	18.2(7.0)	154	12.8(5.6)	66	8.0(3.6)	21	5.5(2.3)	6	4.3(1.4)	12	4.1(1.4)

* D = 'digitisable' arteries, T = total population

+ n = number of arteries

possible to carry out a Student's t-test only one significant difference between 'digitisable' arteries and total population was found with respect to the media (subject 2, uninjected lung, size group 200 to 299μm). With regard to the intima, 4 signficant differences were found between 'digitisable' arteries and the total population, all in the uninjected lungs (subjects 1 and 3, size group ⩾500μm, subject 4, size groups <100μm and ⩾500μm).

Although the technique for obtaining measurements of medial and intimal area and total length of internal elastic lamina using a digitiser was intended for use on uninjected arteries, injected arteries were included in several of the foregoing sections, 2.4.3 - 2.4.5. It was necessary to establish that comments made with regard to reproducibility of measurements, selection of arteries for measurement, and comparison of measured arteries with the total population were equally applicable to uninjected and injected arteries. The reasons for this relate to section 2.4.7 in which measurements of injected and uninjected arteries are compared to assess the effect of arterial distension.

2.4.6 Relationship between Medial Area and Artery Size

The histological sections of all 13 subjects were scanned and each muscular pulmonary artery considered 'digitisable' was measured using Program 1. The relationship between medial area and artery size (total length of internal elastic lamina) was then investigated in each subject using the statistical program 'Simple Regressions'. For all subjects the function giving the best fit between these two parameters was $y = Ax^b$, illustrated for subject 1 in Figure 2.14.

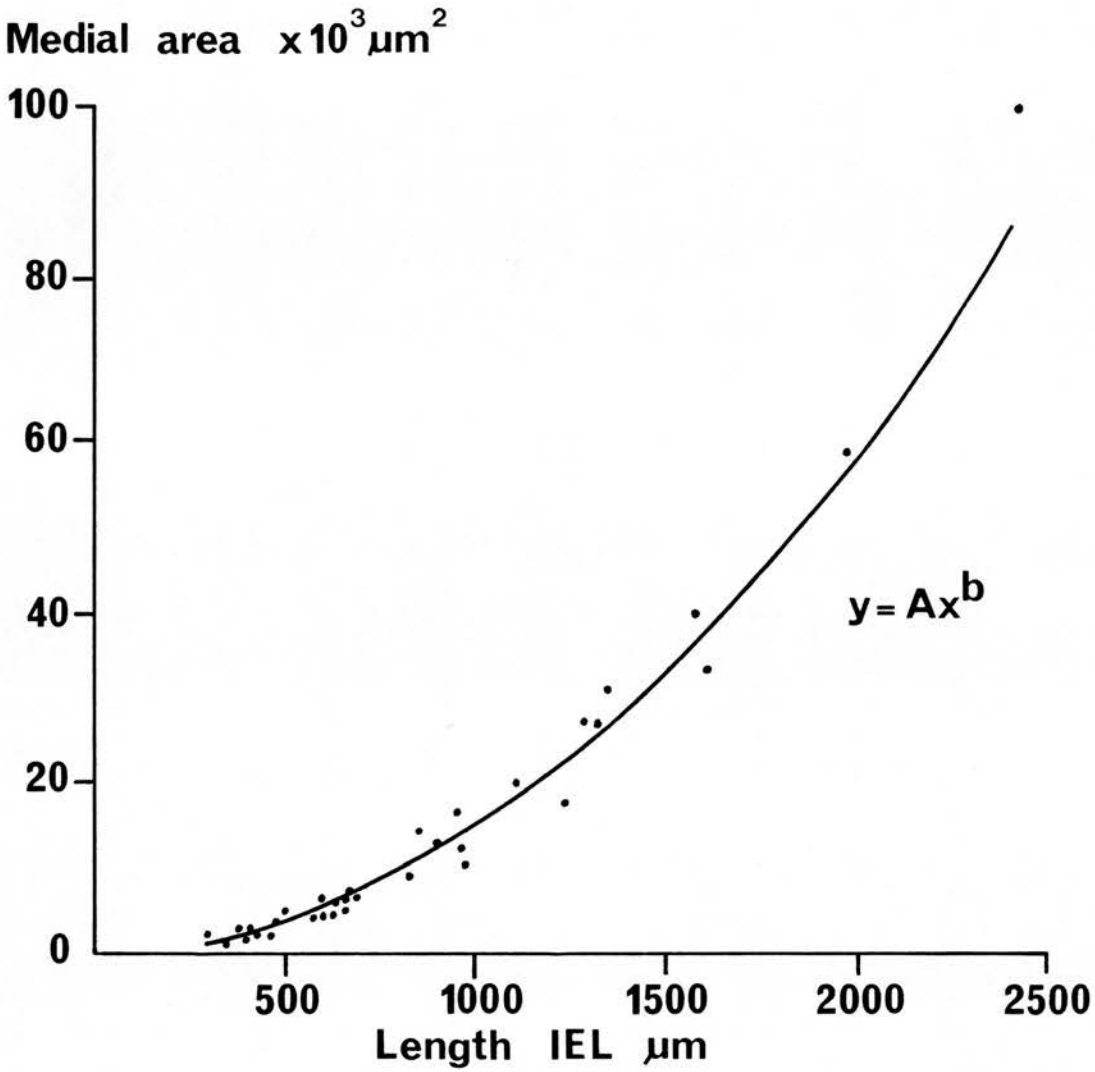


Figure 2.14 The relationship between medial area and length of internal elastic lamina for subject 1.

Such a relationship creates problems when sets of data are to be compared, which may be overcome by linearising the relationship. Three different methods of linearising the relationship were tried:-

1. log medial area plotted against length of internal elastic lamina.
2. log medial area plotted against log length of internal elastic lamina.
3. square root of medial area plotted against length of internal elastic lamina.

All three plots were done using the statistical program 'Simple Regressions' and the function giving the best fit recorded. The results for the 13 subjects were similar and only those of subject 1 are illustrated (Figures 2.15, 2.16 and 2.17). From these figures it can be seen that the best linear fit was achieved by plotting the square root of the medial area against the total length of the internal elastic lamina. It was then possible to compare any two sets of data by assessing whether the slopes of the two regression lines were the same or not, specifically by testing the hypothesis that there was a common slope. In view of the 'tightness' of the relationship between medial area and artery size it was decided to use this particular approach as a means of assessing the effect of different tissue preparation methods on measurements of muscular pulmonary arteries. In so doing, it was intended to include only those arteries which were of comparable size in each pair of tissue preparation groups; there was to be no extrapolation of regression lines.

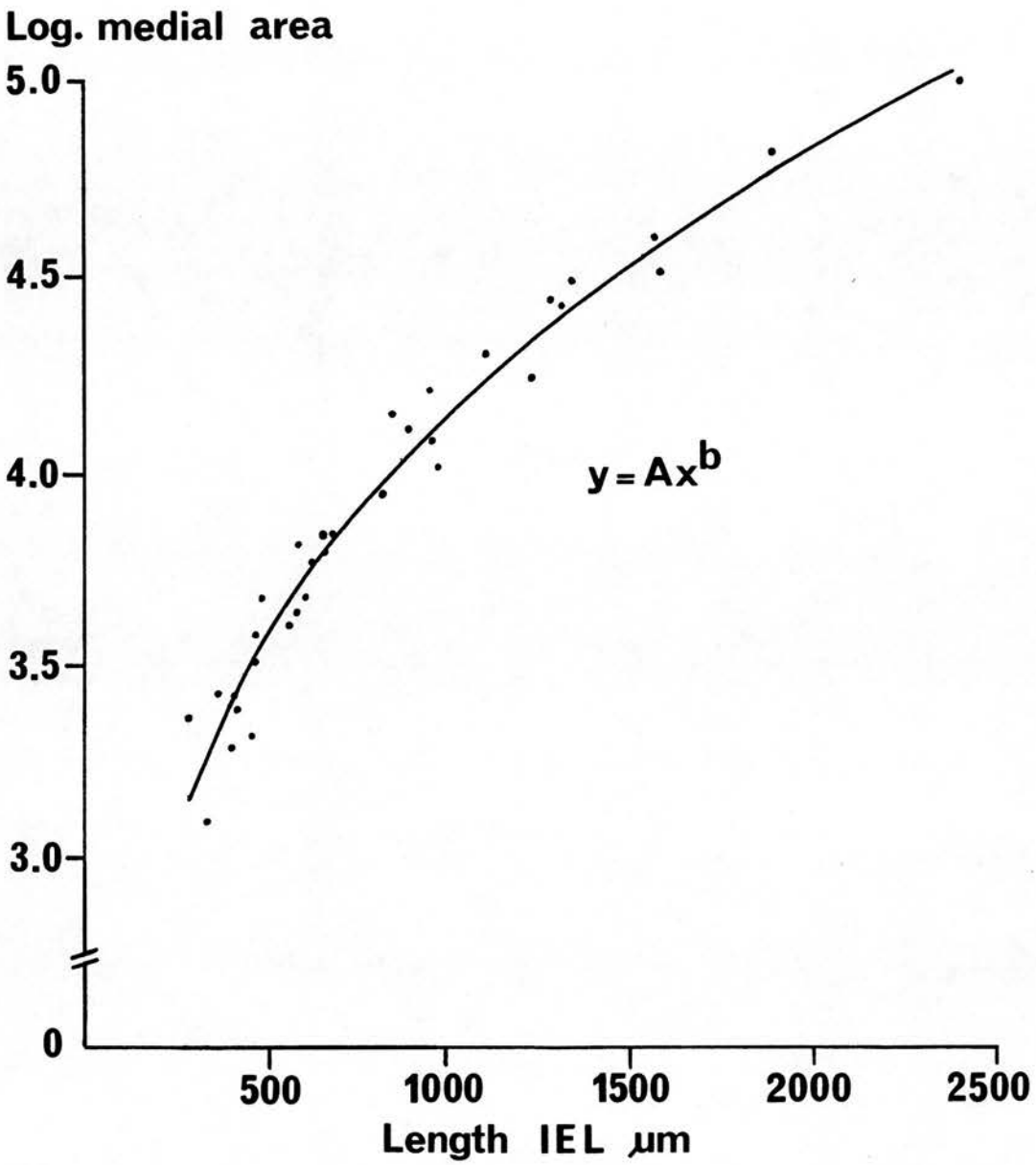


Figure 2.15 The relationship between log. medial area and length of internal elastic lamina for subject 1.

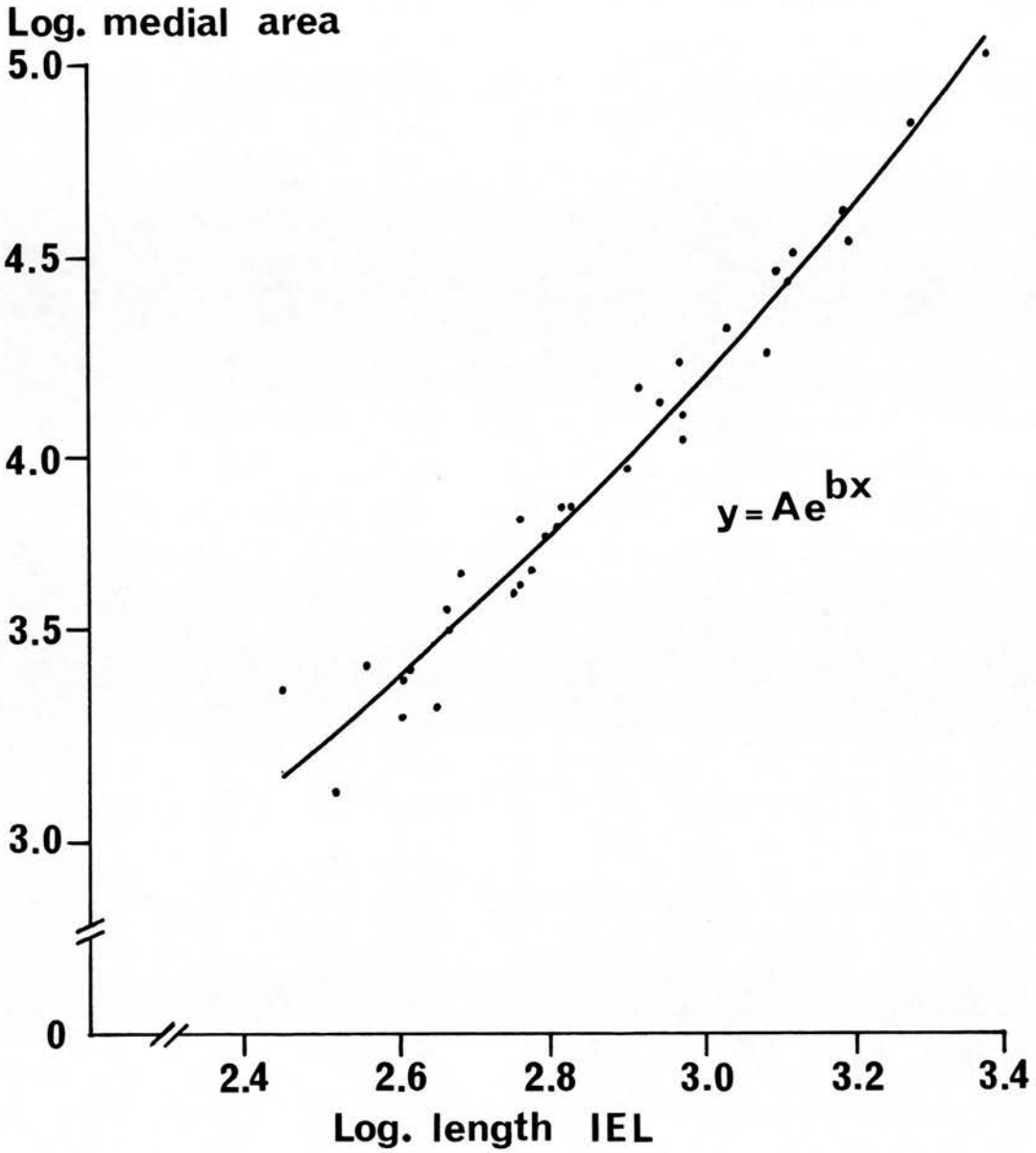


Figure 2.16 The relationship between log. medial area and log. length of internal elastic lamina for subject 1.

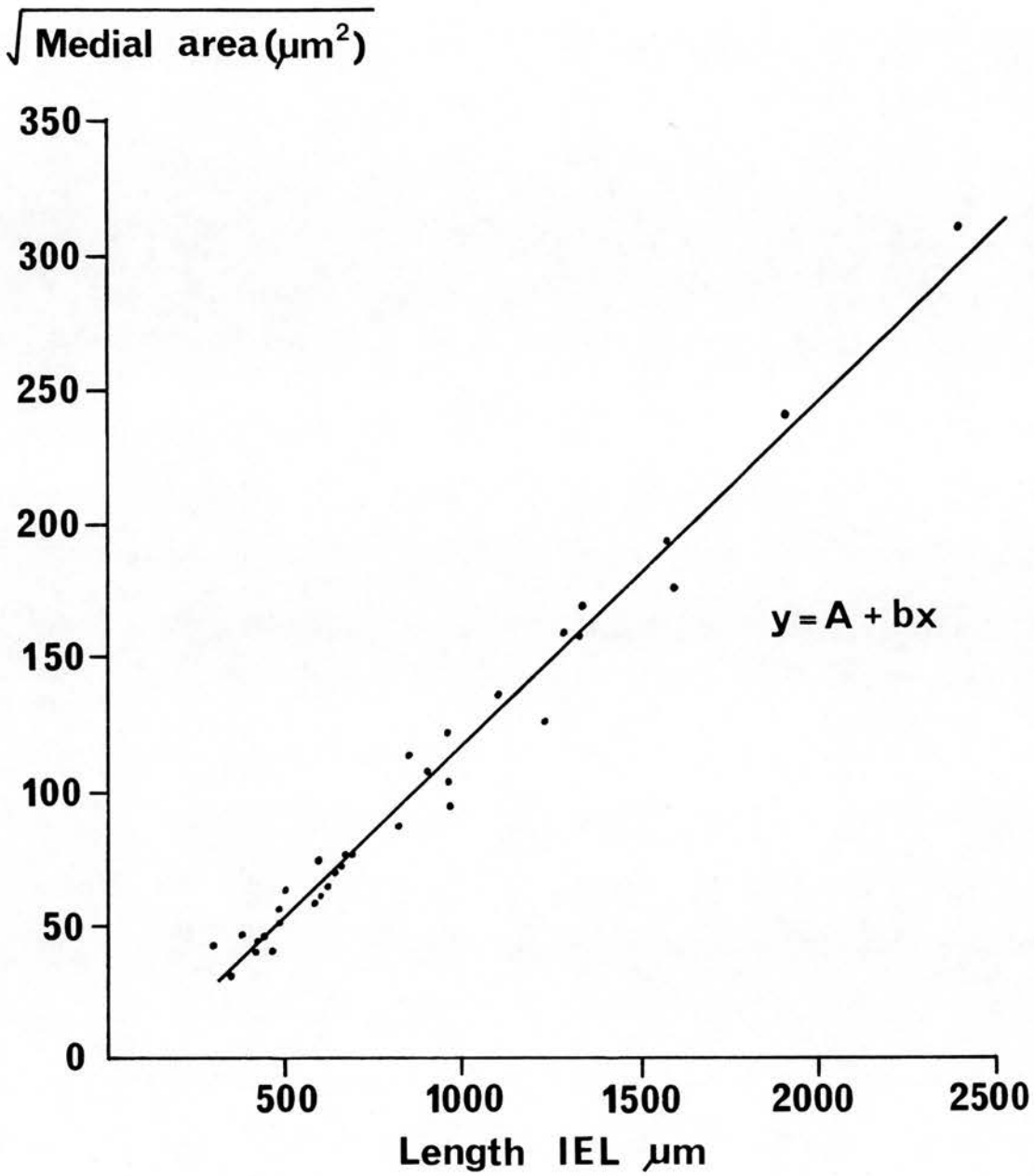


Figure 2.17 The relationship between square root of medial area and length of internal elastic lamina for subject 1.

2.4.7 Comparison of Injected and Uninjected Muscular Pulmonary Arteries

For four subjects (1-4, Table 2.1) the pulmonary arteries of one lung were distended with an injection medium using the method described in section 2.3.2, the other lung was left uninjected. Following tissue fixation and processing the histological sections of the four subjects were scanned and all 'digitisable' arteries measured using Program 1. Using the statistical program 'Simple Regressions' the lines of best fit between square root of medial area and total length of internal elastic lamina were obtained for injected and uninjected arteries. The slopes of the regression lines were compared as described in section 2.3.16.

Although differences were observed between subjects 1, 2, 3 and 4 in terms of medial area associated with an artery of a given size all four subjects showed the same striking difference in the slopes of the regression lines between injected and uninjected arteries illustrated for subject 1 in Figure 2.18. This difference was statisically significant for all four subjects ($p < 0.001$). The difference was such that the internal elastic lamina appeared to have stretched by a factor of approximately 1.5 during the injection process.

2.4.8 Comparison of Muscular Pulmonary Arteries from Routinely Inflated and Uninflated Lungs

For two subjects (8 and 9, Table 2.1) one lung was not inflated, and the other inflated by instillation of formol saline

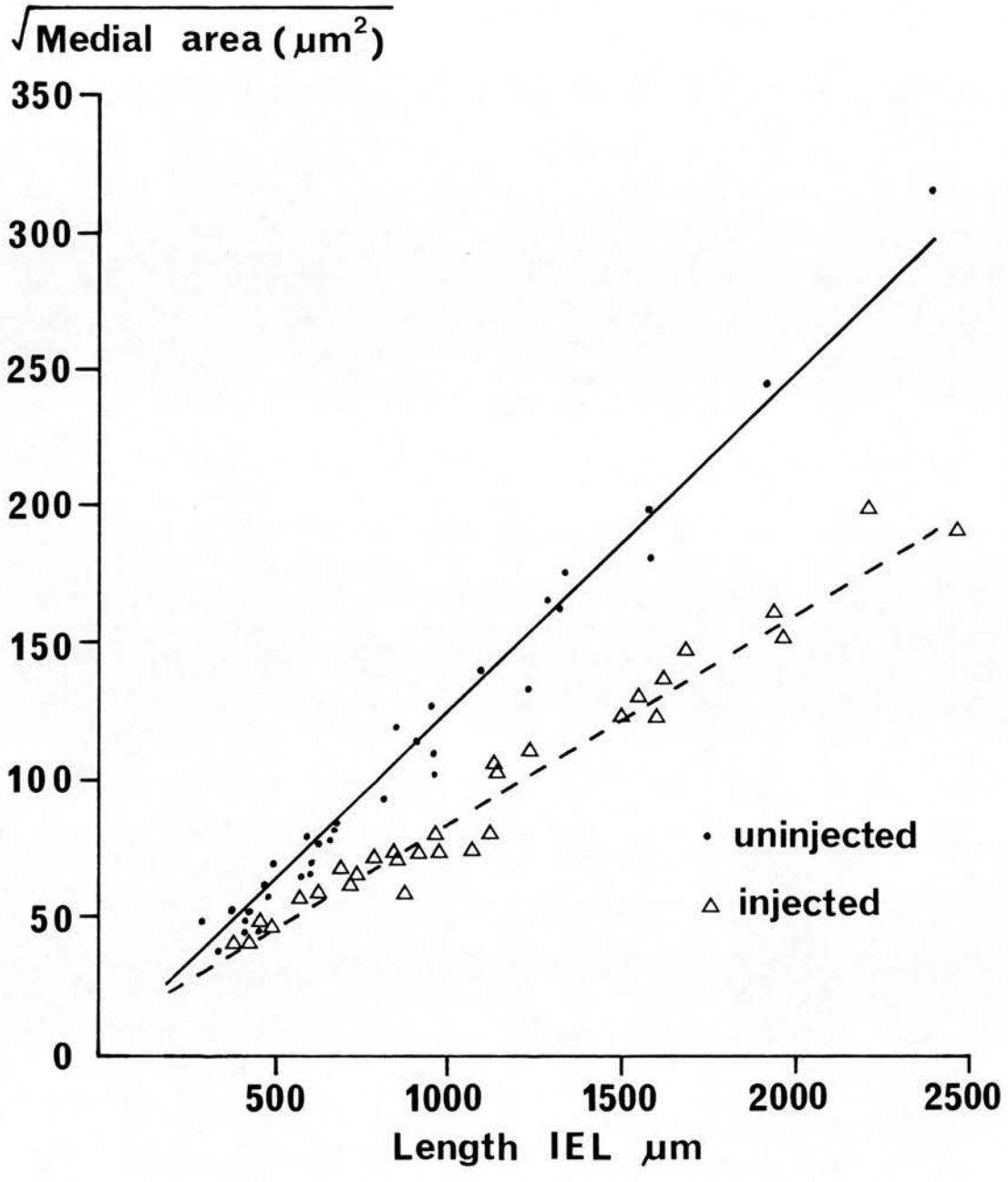


Figure 2.18 The relationship between medial area (square root of) and length of internal elastic lamina in uninjected (-) and injected (- -) muscular pulmonary arteries.

The lines of best fit are:

Uninjected $y = -2.112 + 0.126x$ No. arteries = 33 $r = 0.99$

Injected $y = 6.292 + 0.078x$ No. arteries = 30 $r = 0.98$

into the main bronchus as described in section 2.3.3.

Measurements of 'digitisable' muscular pulmonary arteries were obtained using Program 1 and the data analysed as described in the previous section.

Measurement of arteries by the described technique was possible even using uninflated material. It can be seen from the results of subject 8, illustrated in Figure 2.19, that in general the muscular pulmonary arteries in the uninflated lung appear to have more muscle in their walls than those from the routinely inflated lung. This was evident for all sizes of artery. The difference between uninflated and routinely inflated was not, however, significant. It could, therefore, be assumed that the two regression lines had a common slope.

The results for subject 9 followed an identical pattern.

2.4.9 Comparison of Muscular Pulmonary Arteries from Constant Pressure Inflated and Routinely Inflated Lungs

Using pairs of lungs, one was inflated at constant pressure, the other inflated routinely, both methods as described in section 2.3.3. Measurements of 'digitisable' muscular pulmonary arteries were made according to Program 1.

The slope of the regression line between medial area (square root of) and total length of the internal elastic lamina was the same for the arteries from the routinely inflated and constant pressure inflated lungs. This was the case in both subjects studied

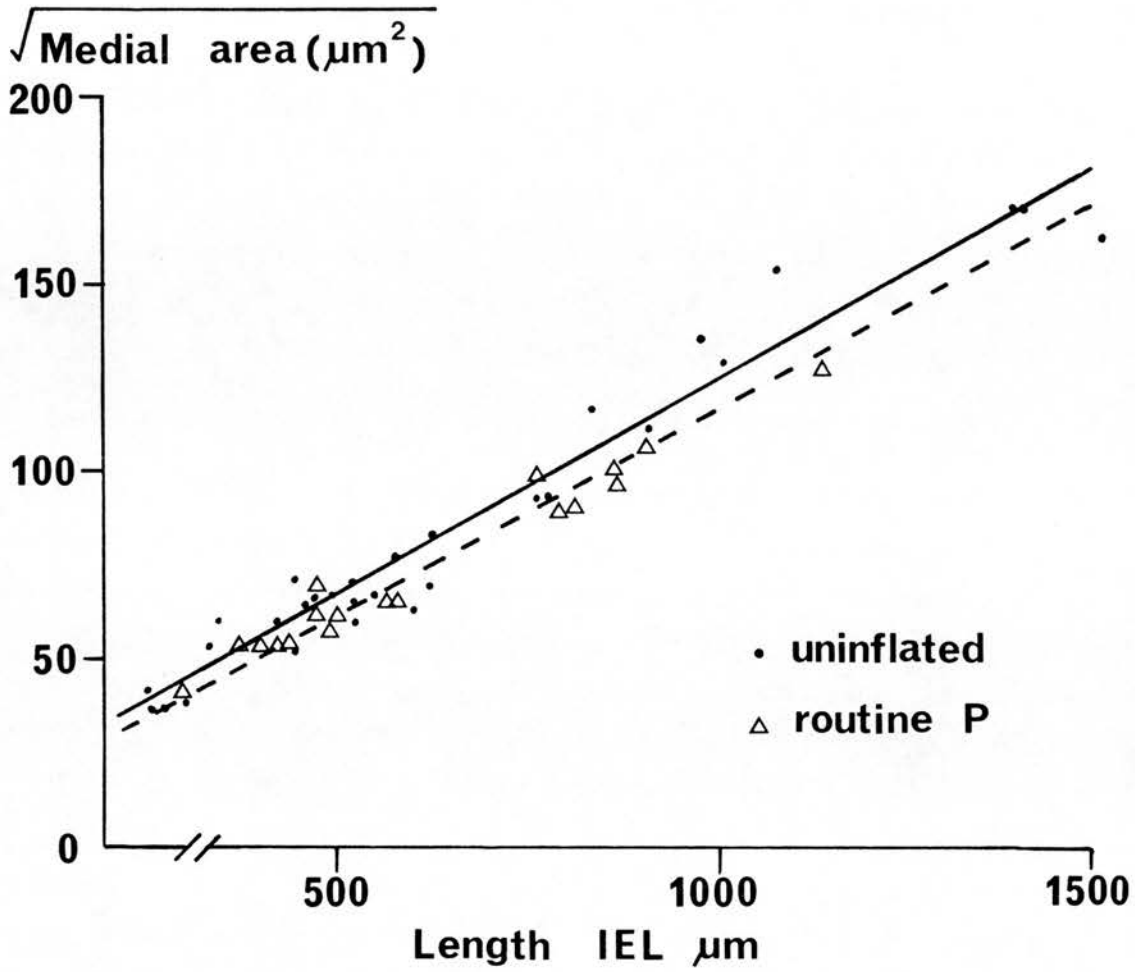


Figure 2.19 The relationship between medial area (square root of) and length of internal elastic lamina in muscular pulmonary arteries from uninflated (-) and routinely inflated (- -) lungs.

The lines of best fit are:

Uninflated	y = 12.114 + 0.115x	No. arteries = 30	r = 0.98
Routinely inflated	y = 8.764 + 0.110x	No. arteries = 18	r = 0.97

(10 and 11, Table 2.1). Figure 2.20 illustrates the results for one of these subjects, subject 11.

2.4.10 Tissue Shrinkage and Compression Resulting from Embedding and Sectioning in Paraffin and Glycol Methacrylate

The tissue blocks taken from three subjects (10, 11 and 12, Table 2.1) were measured before embedding in paraffin or glycol methacrylate, and after embedding and sectioning. Details of the procedure for assessing the amount of shrinkage and compression may be found in section 2.3.8. Data for the individual tissue samples of the three subjects were pooled and the mean percentage linear and area losses due to shrinkage and compression calculated for the paraffin and glycol methacrylate embedded samples. Values for the paraffin embedded samples are given in Table 2.15. Shrinkage and compression of samples embedded in glycol methacrylate and sectioned were considerable negligible (less than 1% on average).

2.4.11 Comparison of Muscular Pulmonary Arteries from Paraffin and Glycol Methacrylate Embedded Tissue

Following measurement of the 'digitisable' muscular pulmonary arteries of the three subjects used in the previous section the square root of medial area was plotted against the length of the internal elastic lamina for the paraffin and glycol methacrylate embedded tissue of each subject. These regressions revealed no consistent or significant differences between the muscular pulmonary arteries in paraffin or glycol methacrylate embedded tissue. This is illustrated for subject 12 in Figure 2.21. However, more

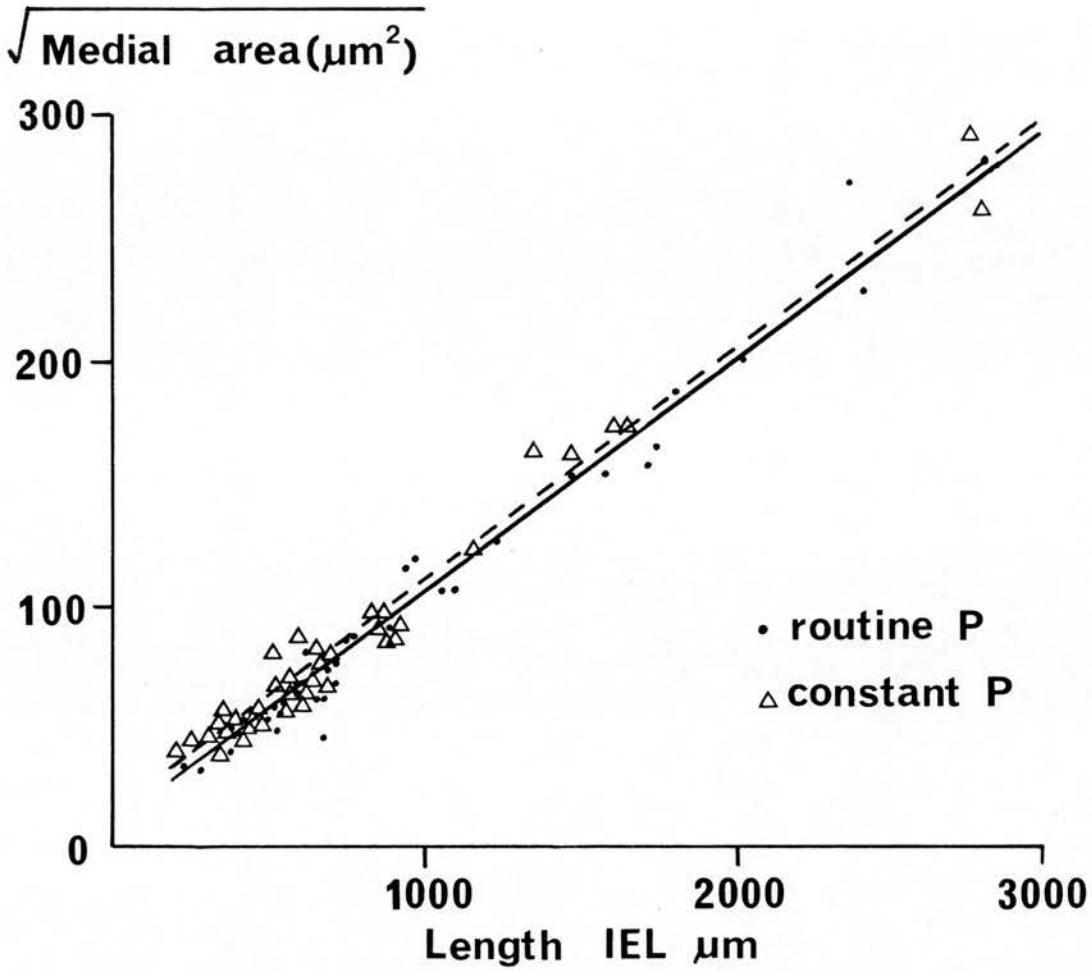


Figure 2.20 The relationship between medial area (square root of) and length of internal elastic lamina in muscular pulmonary arteries from routinely inflated (-) and constant pressure inflated (- -) lungs.

The lines of best fit are:

Routinely inflated	y = 6.126 + 0.096x	No. arteries = 37	r = 0.99
Constant pressure inflated	y = 12.576 + 0.095x	No. arteries = 39	r = 0.99

Table 2.15 Mean* values for percentage linear and area reductions in tissue sample size due to shrinkage and compression in paraffin embedded and sectioned samples. Standard deviations given in brackets.

	Linear loss	Assessed+ area loss	Actual overall area loss
Shrinkage	16% (4.2%)	29%	40% (5.1%)
Compression	7% (3.4%)	11%	

* based on 60 samples

+ Area loss due to shrinkage assessed by taking the square of the linear loss.

Area loss due to compression assessed by subtracting the area loss due to shrinkage from the actual overall area loss.

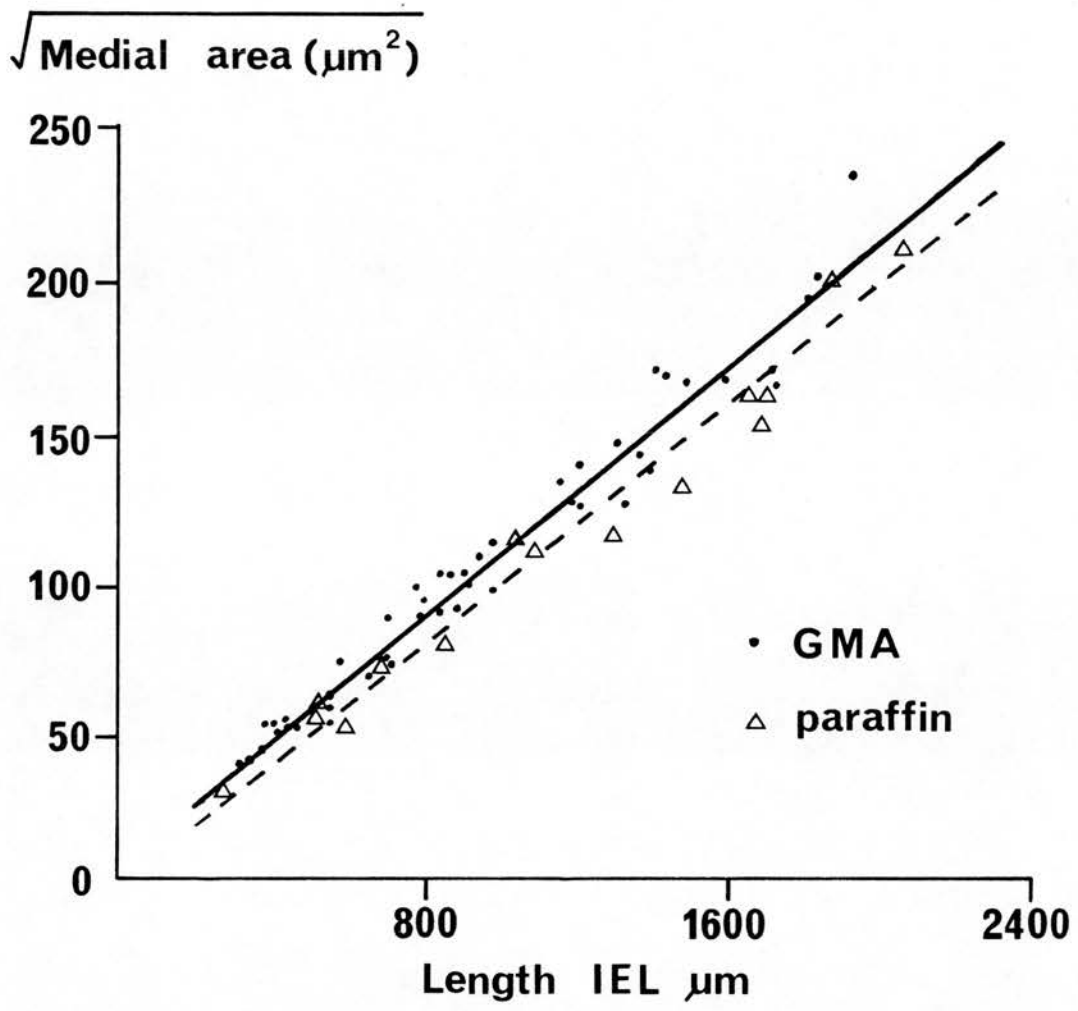


Figure 2.21 The relationship between medial area (square root of) and length of internal elastic lamina in muscular pulmonary arteries from GMA (–) and paraffin (- -) embedded tissue.

The lines of best fit are:

GMA $y = 3.714 + 0.105x$ No. arteries = 47 $r = 0.98$

Paraffin $y = -1.406 + 0.098x$ No. arteries = 15 $r = 0.99$

muscular pulmonary arteries were considered 'digitisable' in glycol methacrylate embedded tissue compared to paraffin embedded tissue, e.g. 47 compared to 15 in subject 12 (illustrated in Figure 2.21).

2.4.12 Reproducibility of Measurements of Intimal Area in Muscular Pulmonary Arteries

This was investigated using 20 muscular pulmonary arteries which covered the full size range of muscular pulmonary arteries and which showed a wide variation in intimal abnormality in terms of type and amount. The arteries also showed a wide variation in the degree of collapse or constriction present. Full details of the subjects from which the arteries were selected is given in section 2.3.19. All arteries were uninjected. Measurements of 11 of the 20 arteries (the uninjected arteries reported on in section 2.4.3) were done using the normal and 'reduced' light emitting diode on the electronic cursor; measurements for the other nine arteries were done using the 'reduced' light source only.

(i) Short-term

For each of the 20 selected muscular pulmonary arteries the mean values of intimal area, measured using Program 1, were calculated from those obtained at three consecutive digitisations; the mean values were considered unimportant and are not quoted. The maximum percentage deviation from the mean was also calculated to provide a measure of the spread of the values. Of the 20 arteries 11 showed deviations of less than 1%, and 16 showed deviations of less than 2% on consecutive digitisations; the maximum percentage

deviation observed was 5.2%. Therefore, few arteries showed more than the expected deviation in view of the results obtained in section 2.4.1.

(ii) Long-term

Using the mean values obtained from the initial consecutive digitisations as a base-line, the long-term repeatability of measurements of intimal area was investigated as described in section 2.3.19. On each of three further digitisations the percentage deviation from the base-line value of intimal area was calculated for each artery. Figure 2.22 shows the maximum percentage deviation observed over the three independent digitising sessions in relation to the base-line value of intimal area. In general, the long-term repeatability of the measurements was excellent with only three of the arteries showing deviations of more than 5% from the base-line value; these were small arteries with a relatively thin intima. One of these arteries is illustrated in Figure 2.23.

(iii) Effect of magnification

The effect of magnification on the measurement of intimal area was investigated in 18 of the 20 muscular pulmonary arteries; these 18 arteries satisfied the criterion of being 'digitisable' at a minimum of three out of four lens objective magnifications: x4, x10, x20, x40. The data are expressed as ratios:-

$$\frac{\text{measurement at specified magnification}}{\text{measurement at x4 magnification}}$$

and are given in Table 2.16. Two points of interest emerge from this table. Firstly, measurement at a magnification of x4 quite

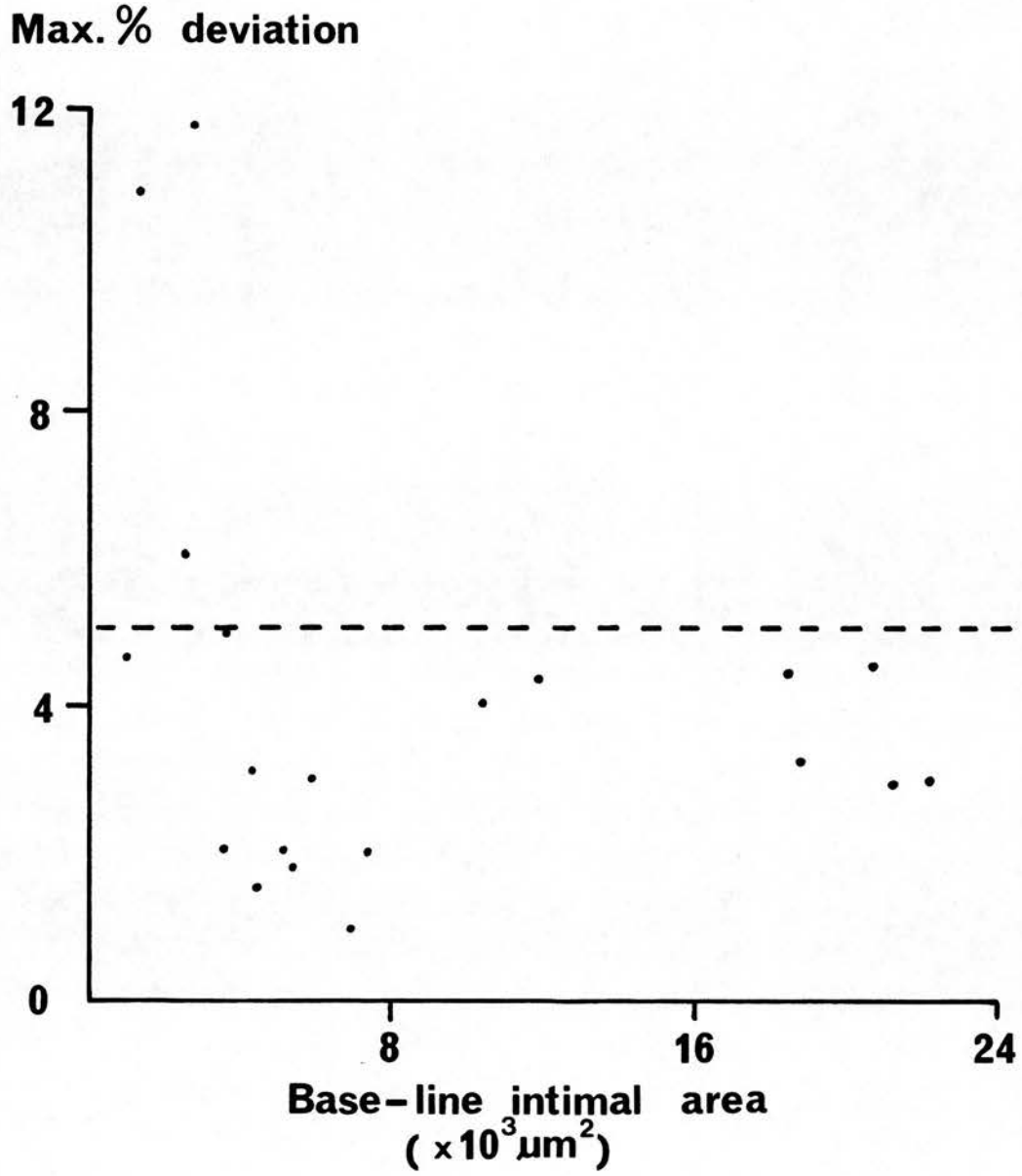


Figure 2.22 The long-term reproducibility of measurements of intimal area expressed in relation to value for intimal area.

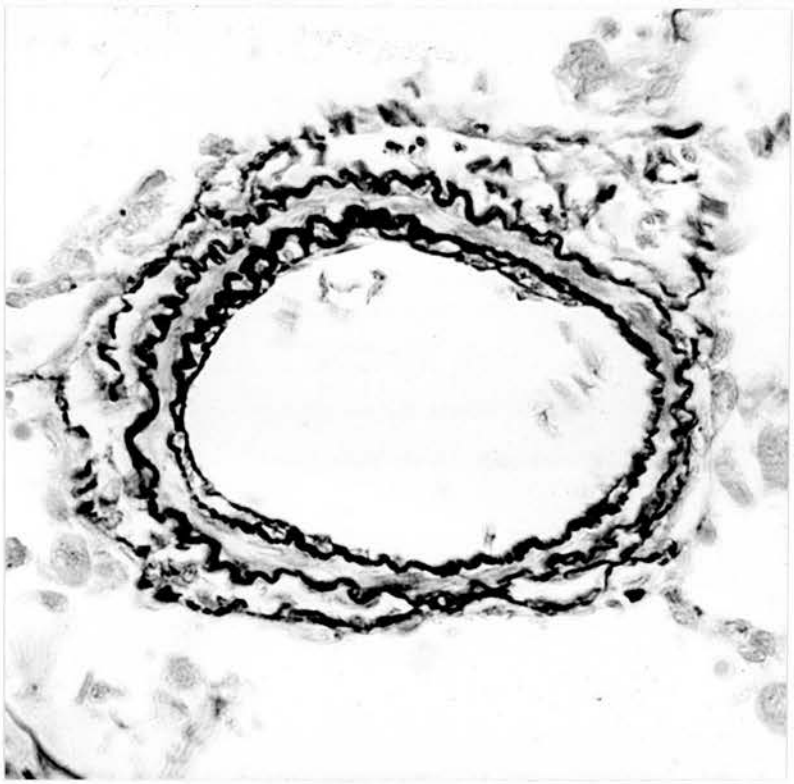


Figure 2.23 Example of one of the small muscular pulmonary arteries with a relatively thin intima.
x 600
Elastic stain

Table 2.16 The effect of magnification on measurements of intimal area in 18 arteries, expressed as measurement at specified lens objective magnification:measurement at x4 magnification.

Artery Number	Specified Magnifications x10	x20	x40
2	0.65	0.61	0.66
3	0.87	0.87	0.93
4	0.92	0.93	-
5	0.93	0.95	-
6	0.88	0.89	0.92
7	1.13	1.18	-
9	0.93	0.96	-
10	0.86	0.83	0.87
11	0.96	0.97	0.98
12	1.04	1.10	-
13	1.02	1.08	1.08
14	1.00	0.93	0.90
15	0.97	1.00	0.98
16	1.02	1.08	-
17	0.97	0.96	0.92
18	0.90	0.93	0.92
19	1.00	1.03	1.04
20	1.08	1.15	1.13

A ratio of 1 throughout for any artery would indicate that the measurement of intimal area was unaffected by magnification.

often produced under- or over-estimations of intimal area which could be quite marked, e.g. arteries 7 and 2 respectively. Secondly, intimal area measurements did not alter much with magnification from x10 upwards. This problem was also present with some medial area measurements (see Table 2.11) but to a much smaller extent. A probable explanation is the relatively greater value for medial area compared with intimal area in most arteries; the smaller the area the more likely that small errors in measurement will seem disproportionately large.

2.4.13 Relationship between Area of Intima and Artery Size

The purpose of this and the following section was to determine what was the best way of expressing the data on intimal abnormality and how best to compare different subjects.

Six subjects (1, 4-7 and 13, Table 2.1) were selected for inclusion in this aspect of the study because they covered a wide range of pathological conditions and intimal abnormality. Only one lung from each subject was studied: subject 1 - right, subject 4 - right, subject 5 - left, subject 6 - left, subject 7 - left, subject 13 - right. Histological sections from the six subjects were scanned and each muscular pulmonary artery considered 'digitisable' was measured using Program 1. The relationship between intimal area and size of artery (defined in terms of total length of internal elastic lamina) was then investigated in each subject using the statisical program 'Simple Regressions'.

It was found that for any size of artery the values for intimal area within all six subjects varied enormously, emphasising the generally irregular distribution of intimal abnormality. Not surprisingly, in view of this, the functions giving the best fit between intimal area and artery size were found to vary. For the six subjects studied three different best fit functions were observed; these are illustrated in Figures 2.24 - 2.26. The observed increase in intimal area with increasing artery size was as expected and was evident in all six subjects.

That no single function would describe the relationship between area of intima and artery size was in marked contrast to the relationship observed between medial area and artery size in section 2.4.6, which was always of the same form, $y = Ax^b$. It was impossible, therefore, to think of comparing subjects by assessing whether the slopes of the relationship between area of intima and artery size were the same or not.

2.4.14 Relationship between Intima Index and Artery Size

One disadvantage of relating area of intima to artery size was that it was not immediately obvious which arteries were most affected by intimal change, in relative terms. It was, therefore, decided to express the data for the six subjects, used in the previous section, in another form. Using the 'Intima Index' program described in section 2.3.14 an Intima Index was calculated for each muscular pulmonary artery, a value of 1 indicating complete occlusion (actual and theoretical) of the lumen. These indices were then plotted against artery size (length of internal elastic lamina)

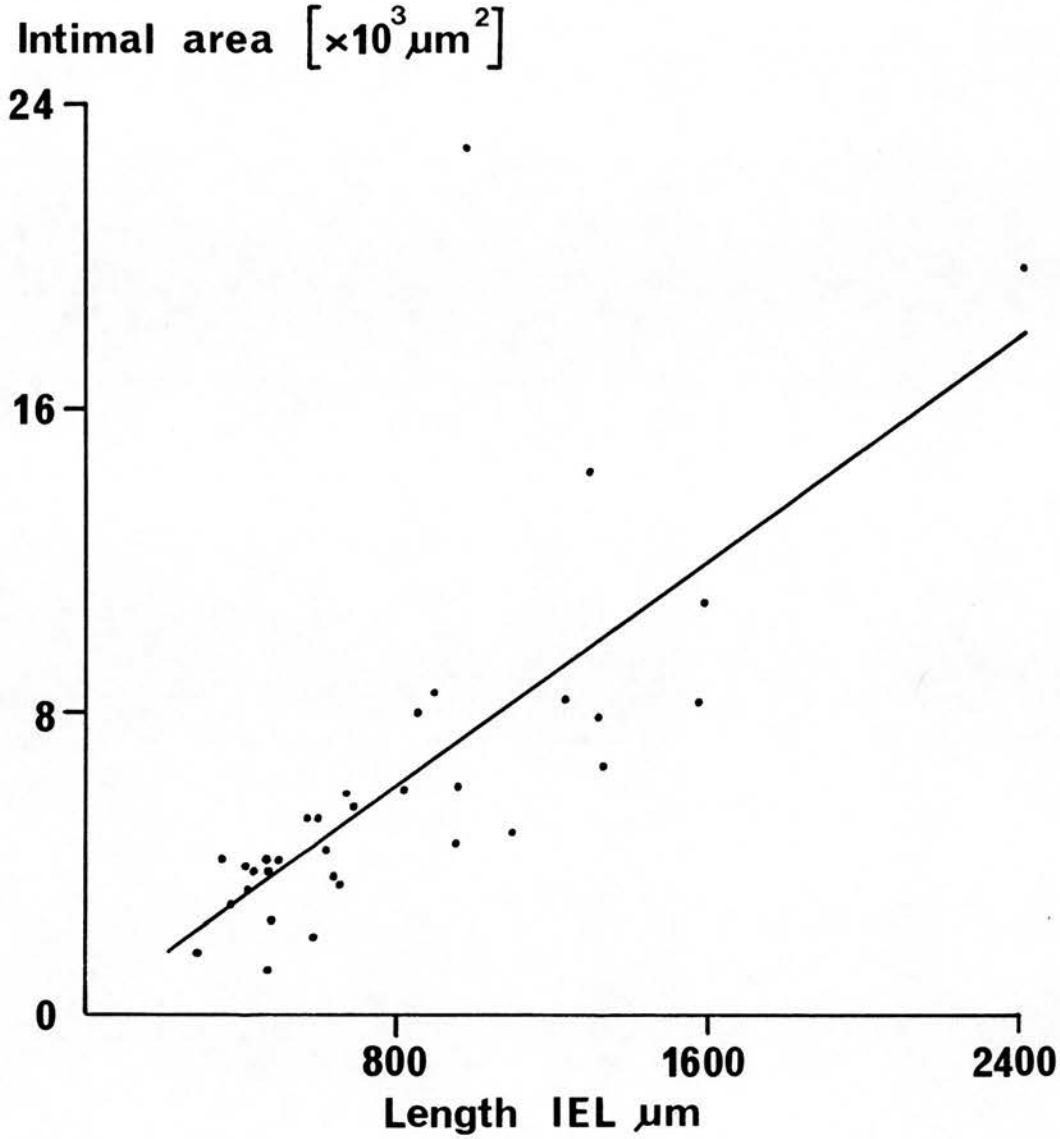


Figure 2.24 The relationship between area of intima and artery size for subject 1.

$y = A + Bx$, $A = 137.15$, $B = 7.53$, $r = 0.73$

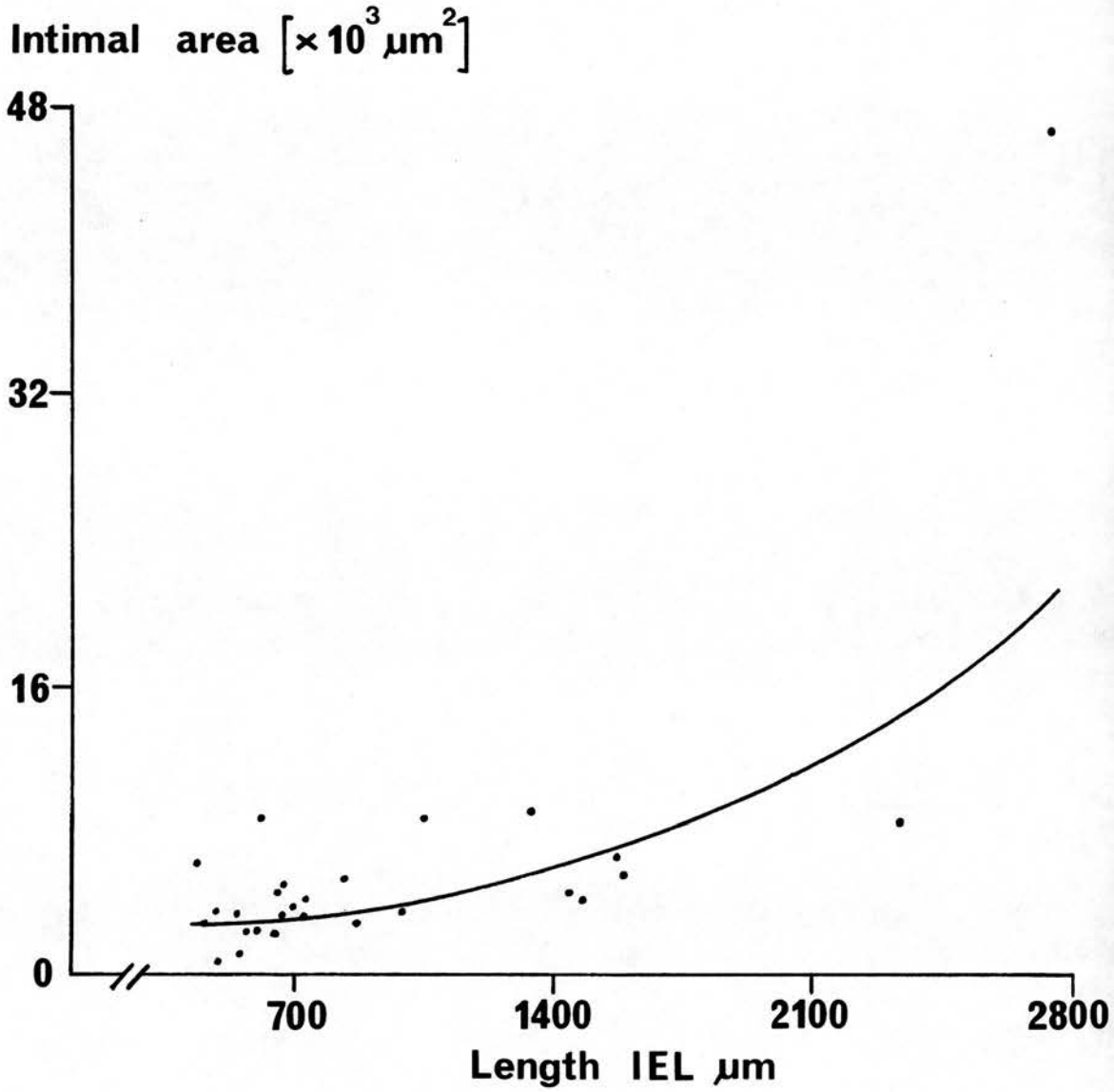


Figure 2.25 The relationship between area of intima and artery size for subject 6.

$y = A.e^{Bx}$ A = 1789.26, B = 0.0009, r = 0.75

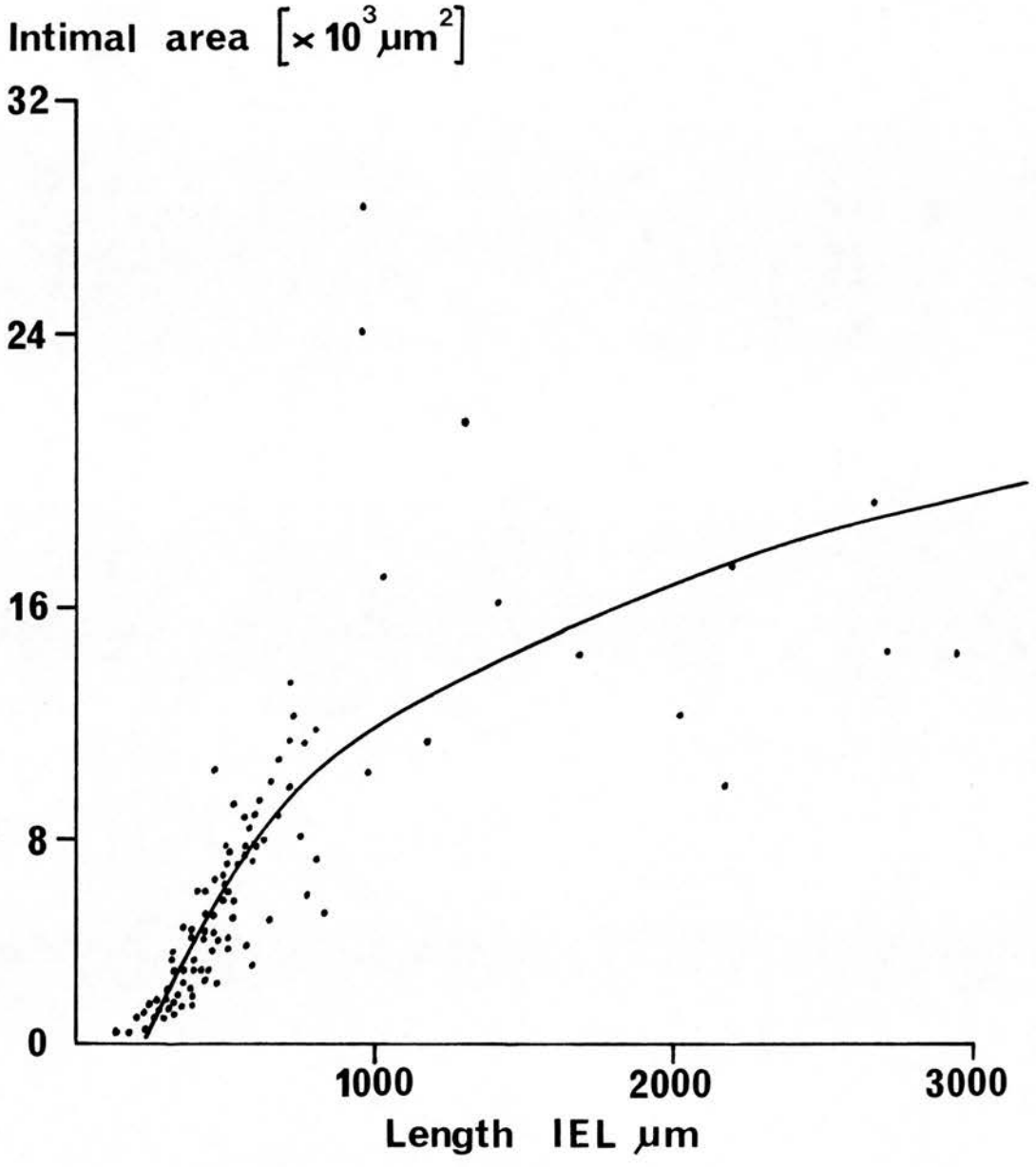


Figure 2.26 The relationship between area of intima and artery size for subject 13.

$y = A + B.\log_e x$ A = -38989.48, B = 7155.30, r = 0.80

for the three subjects (1, 6 and 13) who showed different relationships between area of intima and artery size (see Figures 2.24 - 2.26). It was evident from Figures 2.27 - 2.29 that in all cases the smallest arteries were those most affected by intimal change. There was still no consistent relationship, however, between Intima Index and size of artery in different subjects. It was, therefore, concluded that the most sensible method of comparing intimal abnormality in different subjects would be to calculate a mean Intima Index for arteries sub-divided by size (length of internal elastic lamina); the latter is obviously essential since the smaller arteries are those most affected by intimal change.

2.4.15 Maximising Measurements of Muscular Pulmonary Arteries

For some of the subjects included in this chapter the number of muscular pulmonary arteries considered 'digitisable' (cut in cross-section with a well-defined internal elastic lamina) was few. With regard to the assessment of intimal abnormality in particular, which is often patchy in distribution, it was considered that it would be advantageous if the cross-sectionally cut 'undigitisable' arteries could also be measured. It was thought that this might be possible while continuing to define artery size in terms of the total length of the internal elastic lamina. What was envisaged was obtaining measurements of medial and intimal area by simply delineating the boundaries of these two components, ignoring the crinkles in the elastic laminae. Furthermore, it was thought that it might be possible to estimate the length of an internal elastic lamina by any one of the following three methods:-

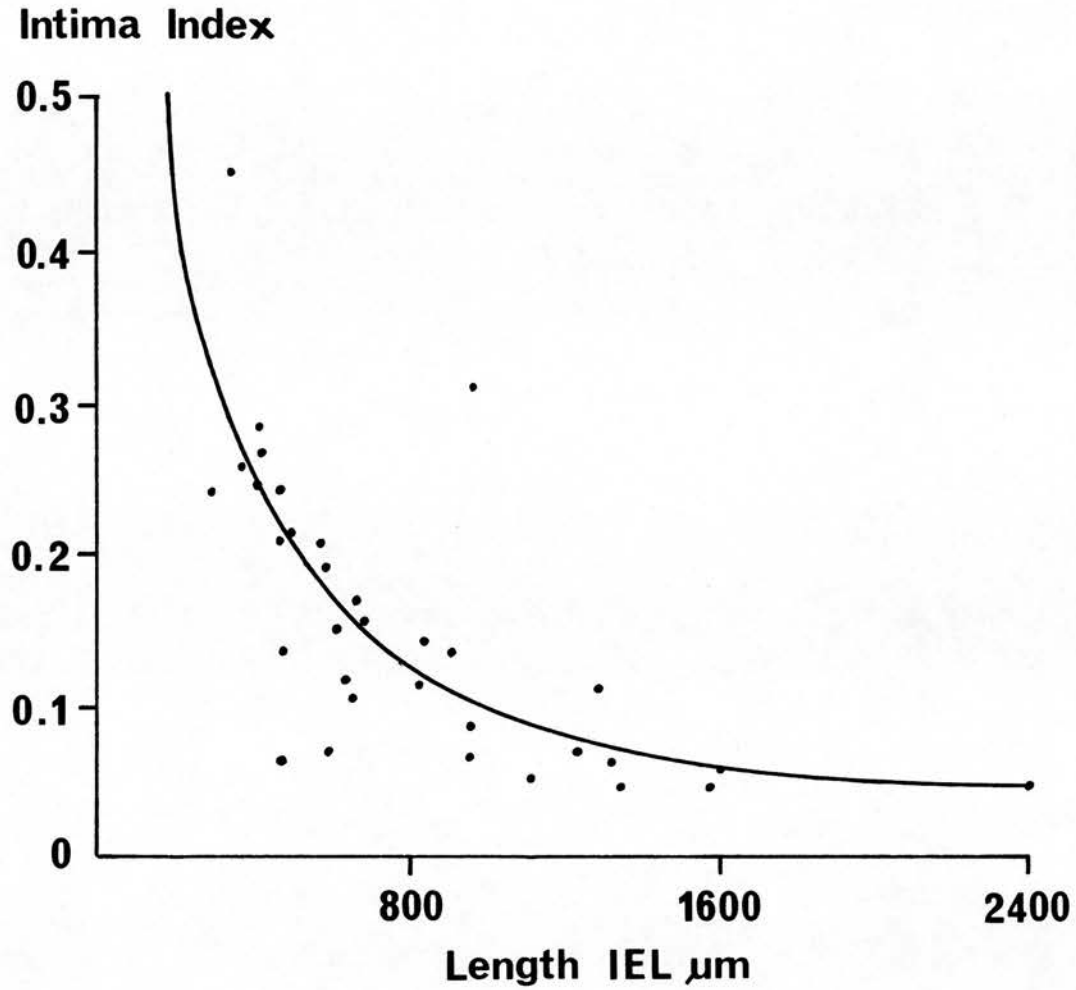


Figure 2.27 The relationship between Intima Index and artery size for subject 1.

$y = A + {}^{B}/x$ A = 0.003, B = 94.21, r = 0.74

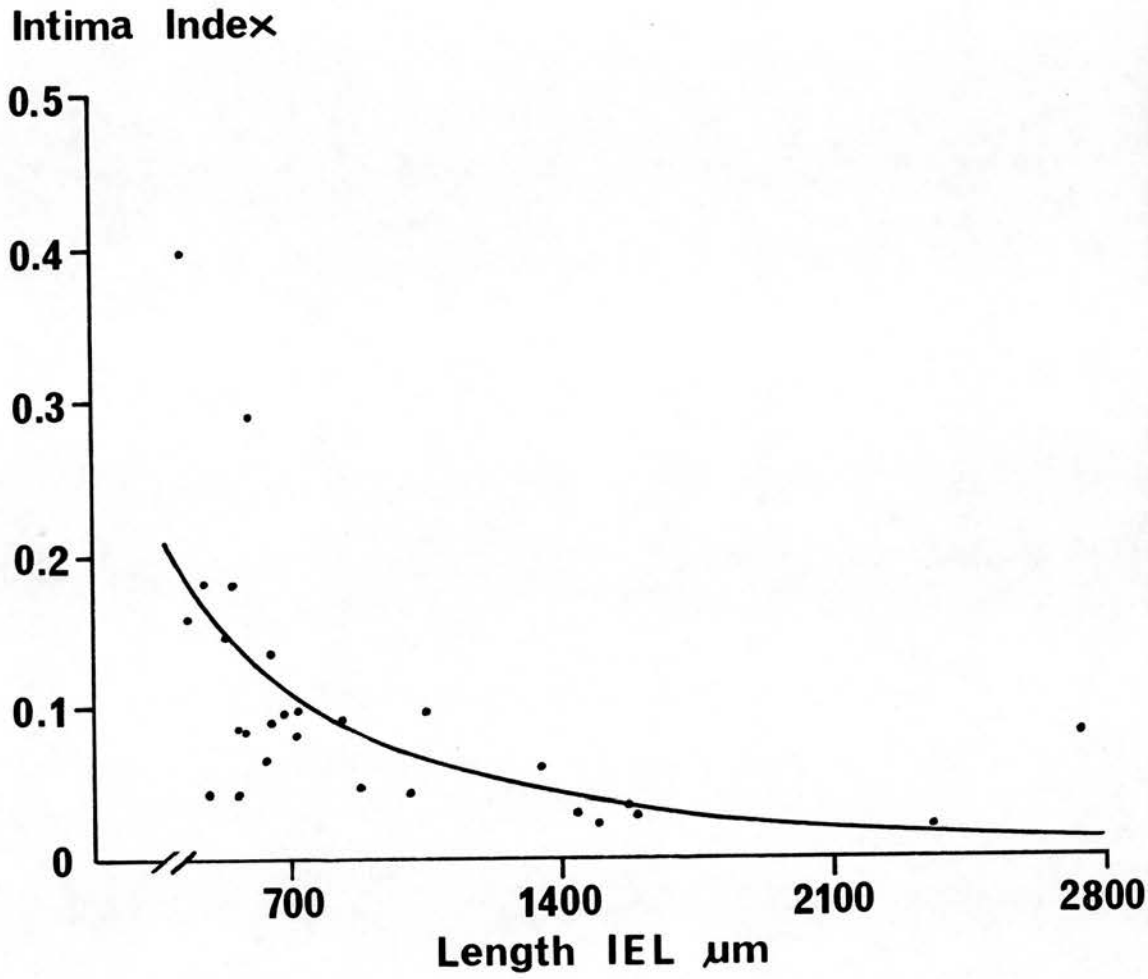


Figure 2.28 The relationship between Intima Index and artery size for subject 6.

$y = A + {}^{B}/x$ A = -0.03, B = 95.80, r = 0.62

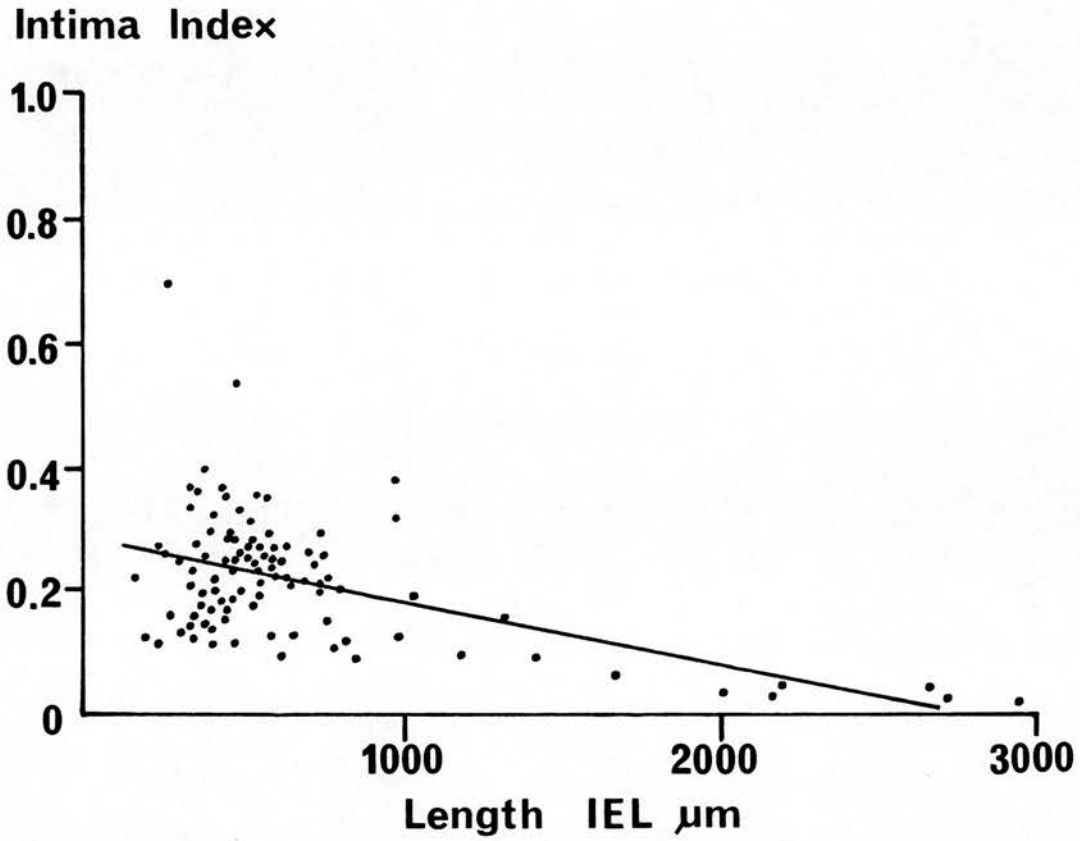


Figure 2.29 The relationship between Intima Index and artery size for subject 13.

$y = A + Bx$ $A = 0.28$, $B = -0.0001$, $r = 0.51$

1. Multiplying the length of the boundary between the intima and media by a factor based on a by-eye estimate of the degree of crinkling (collapse/constriction) in the internal elastic lamina - crinkle grading method.

2. Multiplying the length of the boundary between the intima and media by a factor based on the measured amount of crinkling in a segment of the internal elastic lamina - segmental crinkle factor method.

3. Multiplying the length of the boundary between the intima and media by a factor based on the measured amount of overall crinkling in the elastic laminae of 'digitisable' arteries - mean crinkle factor method.

In order to determine whether the proposed methods for estimating medial and intimal area and total length of internal elastic lamina were satisfactory, it was necessary to test them using arteries for which the true values of these parameters were known, i.e. arteries cut in cross-section with a well-defined internal elastic lamina. For one lung (the left) of three subjects (10, 11 and 12, Table 2.1) all muscular pulmonary arteries considered 'digitisable' were measured, once using Program 1 standard procedure, and once using the abridged procedure described in section 2.3.11.

The measurements obtained for each artery from these two measuring procedures were designated as follows:-

Standard procedure

Medial area - termed MA(1)

Intimal area - termed IA(1)

Total length of internal elastic lamina - termed IEL(1)

Abridged procedure

Medial area - termed MA(2)

Intimal area - termed IA(2)

Length of boundary between intima and media - termed IEL(2)

A number of other measurements were required so that the three proposed methods for estimating the total length of an internal elastic lamina could be tested.

The crinkle grading method required a by-eye estimate of the amount of crinkling in each internal elastic lamina. This was done according to the method described in section 2.3.13, each artery being assigned one of five crinkle grades (range 0 - 4).

The segmental crinkle factor method required that the degree of crinkling be measured in different segments of each internal elastic lamina. This was done by sub-dividing the elastic lamina into eight segments and measuring each segment using the 'Trace Lengths' program as described in section 2.3.13. Values for the segmental lengths of the internal elasic lamina, obtained by tracing the crinkles were termed AB(1), BC(1), CD(1) etc. Values for the corresponding segmental lengths of the boundary between the intima and media were termed AB(2), BC(2), CD(2) etc. The segmental crinkle factors were obtained by simply dividing AB(1) by AB(2), BC(1) by BC(2) etc.

The mean crinkle factor method required that the overall degree of crinkling in each internal elastic lamina be measured for the 'digitisable' arteries. This was easily obtained from the measurements made using the standard and abridged procedure and simply involved dividing the total length of the internal elastic lamina, IEL(1) by the length of the boundary between intima and media, IEL(2).

2.4.16 Comparison of Area Measurements Derived Using the Standard and Abridged Techniques Associated with Program 1

Both the medial and intimal area measurements derived using the two techniques were compared. Table 2.17 indicates that for subjects 10 and 12 there was a closer agreement between medial area measurements than between intimal area measurements. With subject 10 the abridged technique produced medial area measurements that were within 10% of the exact values for all but two of the 39 (5%) arteries. In five (13%) of the arteries, however, the intimal area measurements produced by the abridged technique deviated by more than 10% from the exact values. The corresponding figures for subject 12 are one (2%) and seven (14%). The reverse was the case with subject 11 (Table 2.17); none of the arteries measured showed differences between the intimal area measurements derived by the standard and abridged techniques that were in excess of 10%. In contrast six (15%) of the arteries measured showed medial area differences in excess of 10%. This subject showed the most severe intimal thickening of the three subjects studied. It was concluded

Table 2.17 Comparison of standard and abridged techniques for measuring medial and intimal areas. The table shows the number of arteries in each subject in which the measurements produced by the abridged technique differed by 5% or greater from those derived using the standard technique. The figures in brackets are the number of arteries in which the differences were 10% or greater.

Subject Number	No. arteries measured	Intima	Media
10	39	20 (5)	6 (2)
11	41	9 (0)	15 (6)
12	50	20 (7)	7 (1)

that agreement between area measurements derived by the standard and abridged techniques was closer for whichever structural component (media or intima) in that artery encompassed the bigger area.

2.4.17 Comparison of Different Methods of Estimating the Length of an Internal Elastic Lamina

(i) The crinkle grading method

Having graded each artery by eye for degree of constriction/collapse on a five point scale, calculations were done to determine how much longer on average was the total length of the internal elastic lamina (IEL (1)) compared to the boundary between the intima and media (IEL (2)) for arteries with a crinkle grade of 0, 1, 2, 3 and 4 in each subject. With subject 12, for example, it was found that the total length of the internal elastic lamina (IEL (1)) was 23%, 32% and 47% longer than the measurement of the boundary between intima and media (IEL (2)) in arteries graded 1, 2 and 3 by eye respectively. No arteries were graded 0 or 4. Treating each artery as an 'undigitisable' artery attempts were made to estimate the length of its internal elastic lamina by multiplying the measurement IEL (2) by the mean value of 23%, 32% or 47% depending on the crinkle grade assigned to that artery. Table 2.18 shows the estimated values for the lengths of the internal elastic lamina in the arteries of subject 12 together with the measured values, and the percentage errors incurred. In only 13 (26%) of the arteries did the estimated value differ by more than 10% from the measured

Table 2.18 Estimated* and measured values for length of internal elastic lamina in the arteries of subject 12 together with the percentage errors.

Estimated IEL (1)	Measured IEL (1)	% Error	Estimated IEL (1)	Measured IEL (1)	% Error
754.1	818.5	7.9	1788.1	1984.1	9.9
1764.8	1983.3	11.0	1524.4	1568.6	2.8
737.7	801.3	7.9	1359.8	1343.7	1.2
452.6	489.7	7.6	456.2	411.6	10.8
1331.4	1368.3	2.7	1424.1	1585.8	10.2
2512.8	3129.4	19.7	1293.8	1293.6	0.02
1051.3	1027.2	2.4	518.2	489.1	5.9
423.1	420.9	0.5	1067.8	1017.6	4.9
479.7	472.4	1.5	529.6	500.9	5.7
2414.8	2373.7	1.7	1038.7	888.6	16.9
989.5	971.6	1.8	1551.4	1362.7	13.8
461.5	434.9	6.1	2058.0	2211.7	6.9
677.3	689.6	1.8	1903.8	1791.1	6.3
890.4	881.4	1.0	462.7	472.6	2.1
519.9	523.0	0.6	1038.8	1041.6	0.3
1404.7	1421.4	1.2	340.7	328.0	3.9
1519.4	1604.5	5.3	1124.1	1072.5	4.8
828.2	824.3	0.5	405.1	414.7	2.3
2632.2	2943.4	10.6	1505.6	1507.4	0.1
563.5	552.6	2.0	957.1	892.9	7.2
639.3	590.9	8.2	530.2	474.8	11.7
697.1	629.6	10.7	447.6	422.4	6.0
1244.6	1337.0	6.9	1513.5	1700.2	11.0
1525.6	1680.3	9.2	943.0	810.3	16.4
976.7	993.7	1.7	643.1	566.7	13.5

* estimated by the crinkle grading method

value. With subjects 10 and 11 the corresponding figures were 21% and 32%.

On the basis of these results it was concluded that the crinkle grading method for estimating the length of an internal elastic lamina was a practical possibility.

(ii) The segmental 'crinkle factor' method

Table 2.19 lists the range of segmental 'crinkle factors' observed in the arteries of subject 12 together with the overall artery 'crinkle factors'. It is obvious from this table that the degree of constriction in any artery was not uniform round the artery wall. Several arteries showed an increase in 'crinkle factor' between the least and most constricted segments of the internal elastic lamina in the order of 100%. Similar trends were evident in the other two subjects studied.

In view of the considerable range in values for segmental 'crinkle factors' it was concluded that the total length of an internal elastic lamina could not be approximated by multiplying the length of the boundary between the intima and media, IEL (2), by a 'crinkle factor' derived from any one segment of that internal elastic lamina.

(iii) The mean 'crinkle factor' method

For the three subjects the overall 'crinkle factor' of each artery was plotted against its size (defined as IEL (1)). The results are illustrated in Figures 2.30 - 2.32. Two very important points emerge from these figures. For arteries of similar size there was some variation in the overall 'crinkle factors' observed.

Table 2.19 The range of segmental 'crinkle factors' in each artery of subject 12 together with the overall artery 'crinkle factor'.

Range of Segmental CFs*	Artery CF	Range of Segmental CFs	Artery CF
1.13 - 1.47	1.34	1.10 - 2.15	1.47
1.28 - 1.83	1.48	1.26 - 2.34	1.51
1.12 - 2.01	1.43	1.14 - 1.96	1.45
1.18 - 1.82	1.43	1.15 - 1.41	1.19
1.11 - 1.85	1.51	1.46 - 1.90	1.64
1.22 - 1.96	1.64	1.11 - 1.79	1.32
1.36 - 1.95	1.44	1.22 - 1.91	1.39
1.14 - 1.77	1.22	1.29 - 1.85	1.40
1.10 - 1.53	1.30	1.14 - 1.35	1.16
1.03 - 2.00	1.30	1.26 - 1.57	1.26
1.32 - 2.18	1.44	1.09 - 1.38	1.16
1.03 - 2.01	1.24	1.32 - 2.21	1.58
1.01 - 1.92	1.34	1.25 - 1.76	1.38
1.20 - 2.34	1.46	1.36 - 2.10	1.35
1.24 - 2.00	1.33	1.24 - 2.62	1.47
1.15 - 1.96	1.49	1.15 - 1.54	1.18
1.27 - 1.86	1.55	1.31 - 1.86	1.40
1.13 - 1.84	1.46	1.47 - 1.95	1.35
1.19 - 1.95	1.64	1.06 - 2.05	1.47
1.11 - 1.76	1.29	1.18 - 1.81	1.37
1.05 - 1.58	1.22	1.14 - 1.98	1.18
1.20 - 1.47	1.19	1.16 - 1.55	1.25
1.18 - 1.81	1.42	1.24 - 2.67	1.65
1.41 - 1.81	1.62	1.14 - 1.49	1.26
1.32 - 2.03	1.50	1.12 - 1.48	1.16

* CF = Crinkle factor

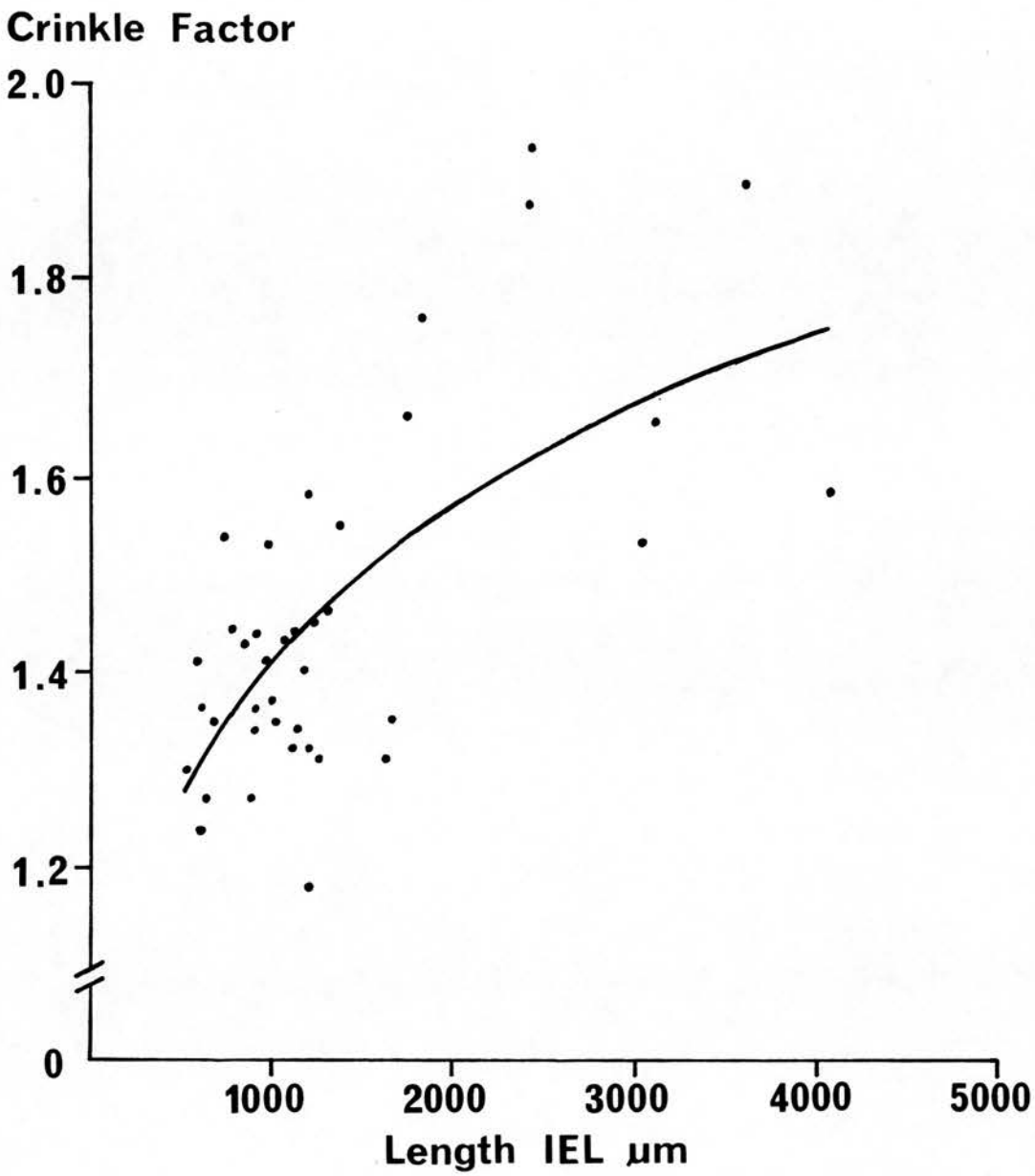


Figure 2.30 The relationship between artery crinkle factor and artery size in subject 10.

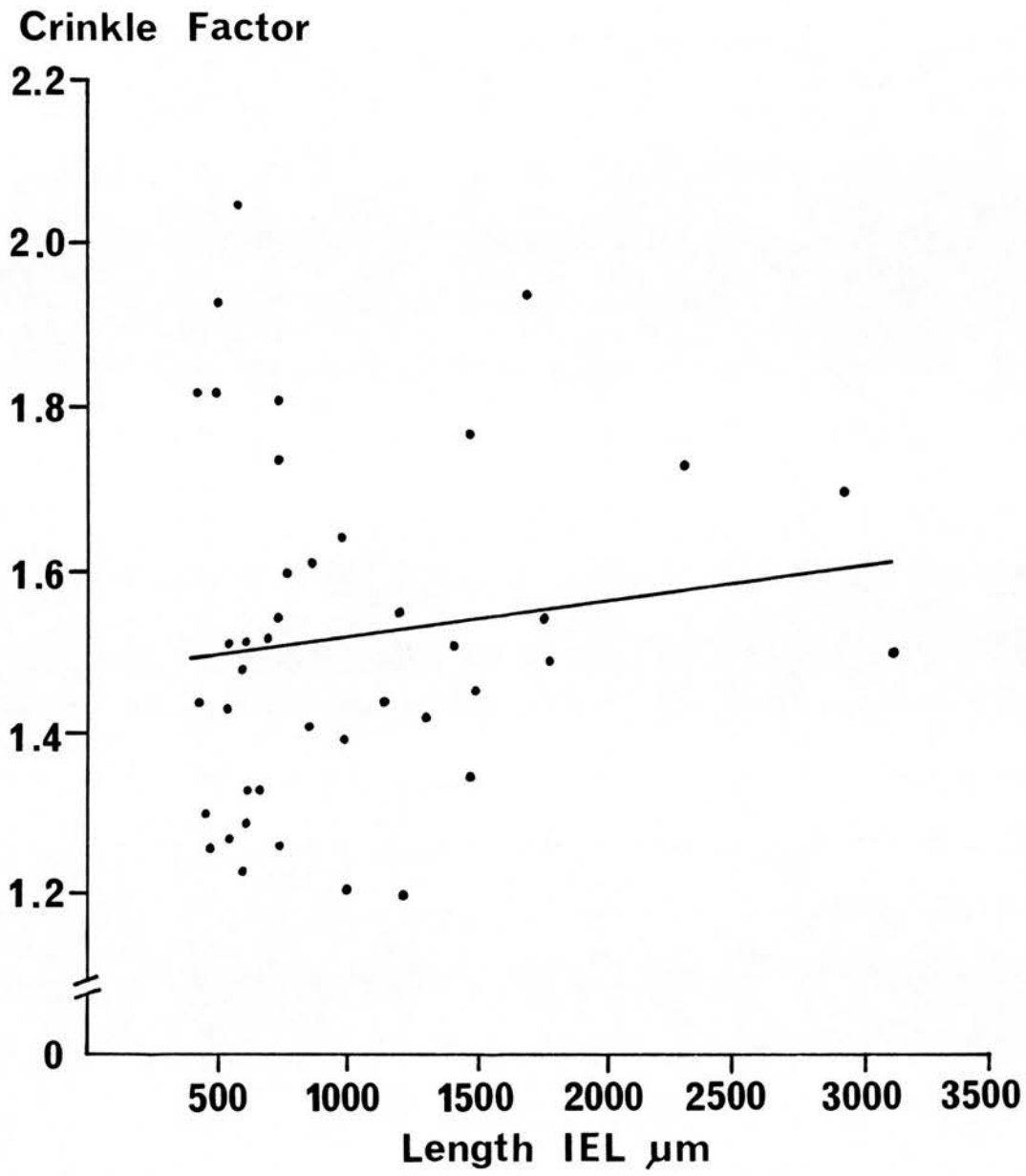


Figure 2.31 The relationship between artery crinkle factor and artery size in subject 11.

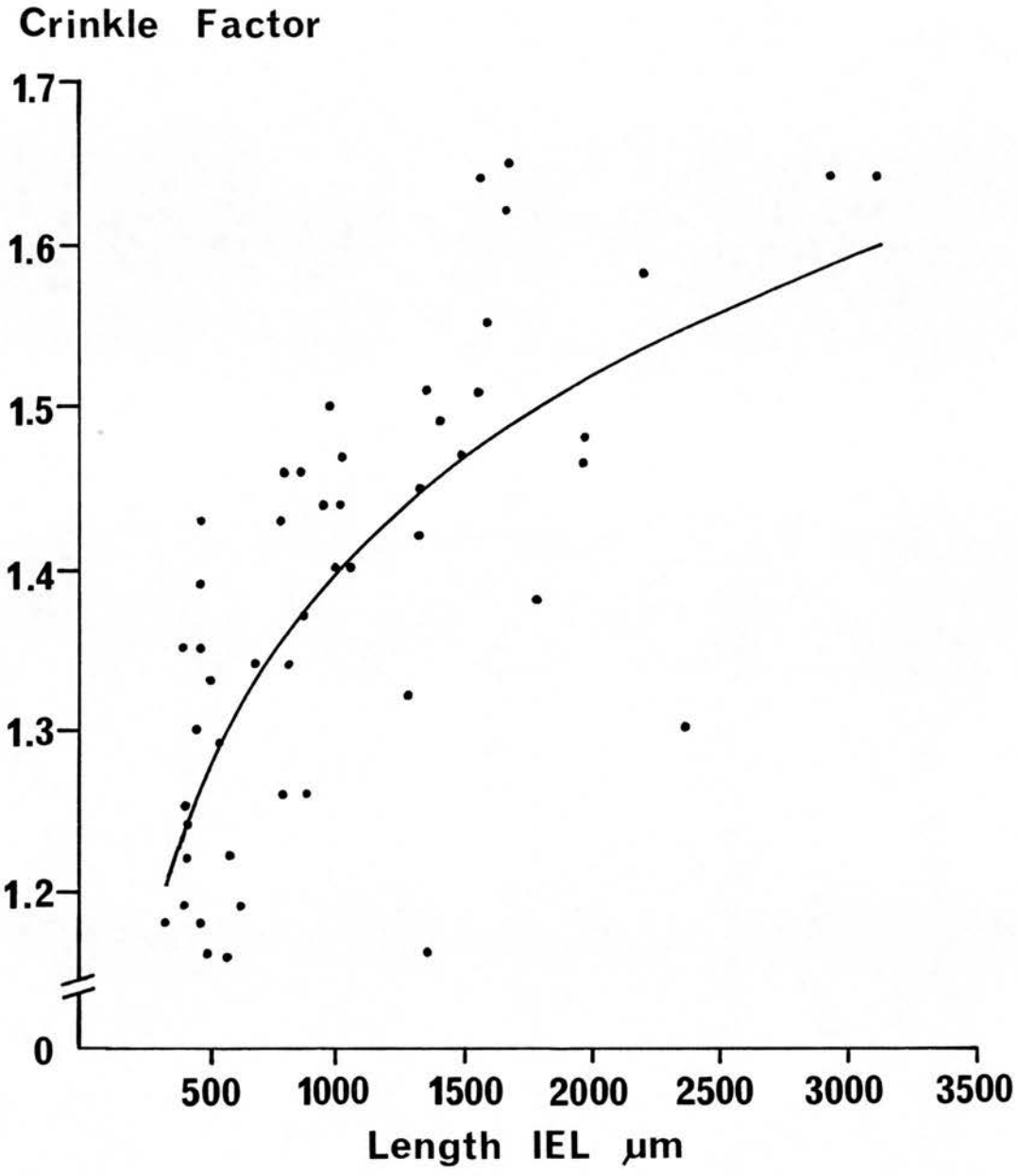


Figure 2.32 The relationship between artery crinkle factor and artery size in subject 12.

However, this difference in 'crinkle factor' between arteries of a similar size was generally less marked than that between segments of a single artery. The second important point is that the 'crinkle factor' tended to increase with increasing size of artery indicating that the larger arteries were more constricted/collapsed. The increase in 'crinkle factor' with artery size was seen in all three subjects but to varying extents. This may have important implications with regard to the commonly used 'wall thickness' methods of measurement.

In effect, these findings ruled out the possibility that the total length of an internal elastic lamina could be estimated by multiplying the measurement IEL (2) by a mean 'crinkle factor' based on the 'digitisable' arteries.

(iv) The size dependent mean 'crinkle factor' method

In theory this method for estimating the total length of an internal elastic lamina might have worked. However, it was not attempted because it was considered too complicated to be of any practical use. It would have involved sub-dividing the 'digitisable' arteries by size and calculating mean crinkle factors (IEL(1) divided by IEL(2)) for each group. To then estimate the total length of the internal elastic lamina in an 'undigitisable' artery would have involved multiplying the length of the boundary between intima and media (IEL(2)) by the appropriate size dependent mean crinkle factor. This was the problem area. The decision on which crinkle factor to apply could not have been made without a knowledge of the true size of the 'undigitisable' artery, namely

total length of internal elastic lamina, the very parameter for which a measurement was trying to be produced.

Up to this point the main concern had been in assessing the errors incurred in the measurement of medial and intimal areas in the individual artery using the abridged technique; these errors were generally less than 10%. In addition, a comparison had been made of three different methods of estimating the length of an internal elastic lamina, one of which (the crinkle grading method) produced estimated values that were within 10% of the actual values for the majority of arteries measured. The problem at this point was how to determine whether the techniques for estimating medial area, intimal area and total length of internal elastic lamina were acceptable or not. It was decided that this problem was best solved by investigating the overall relationships between medial area and artery size, and intimal area and artery size.

2.4.18 The Relationship between Medial Area and Artery Size Using Measured and Estimated Values

In accordance with the results obtained in section 2.4.6 the square root of medial area was plotted against the total length of the internal elastic lamina in all cases to linearise the relationship. A comparison of the slopes of the regression lines was done as described in section 2.3.16 to determine whether the slopes, and hence relationships, were the same or different.

Using the statistical program 'Simple Regressions' analyses were carried out for each subject of the relationship between (a)

$\sqrt{MA\ (1)}$ and IEL (1) and (b) $\sqrt{MA\ (2)}$ and estimated IEL (1) as determined by the crinkle grading method. The relationships (a) and (b) for the three subjects are illustrated in Figures 2.33 - 2.35. For each subject the slopes of the two regression lines were not significantly different and were in most cases virtually identical.

2.4.19 The Relationship between Intima Index and Artery Size Using Measured and Estimated Values

In section 2.4.14 it was concluded that the most readily understandable method of expressing patchy intimal change in a subject was to calculate a mean Intima Index for arteries sub-divided by size. Accordingly, two sets of Intima Indices were calculated for each artery in the three subjects; for Intima Index (1) the Index was calculated from the measurements IA (1) and IEL (1) and for Intima Index (2) from IA (2) and estimated IEL (1) as determined by the crinkle grading method.

Arteries were sub-divided into three arbitrarily selected groups according to the length of the internal elastic lamina, <1000, 1000-2000 and >2000μm. Mean Intima Indices (1 and 2) were then calculated for arteries in those size groups. The values for all three subjects are illustrated in Table 2.20. This table shows that there were no appreciable differences between mean values of Intima Index (1) and (2) in any size group or subject.

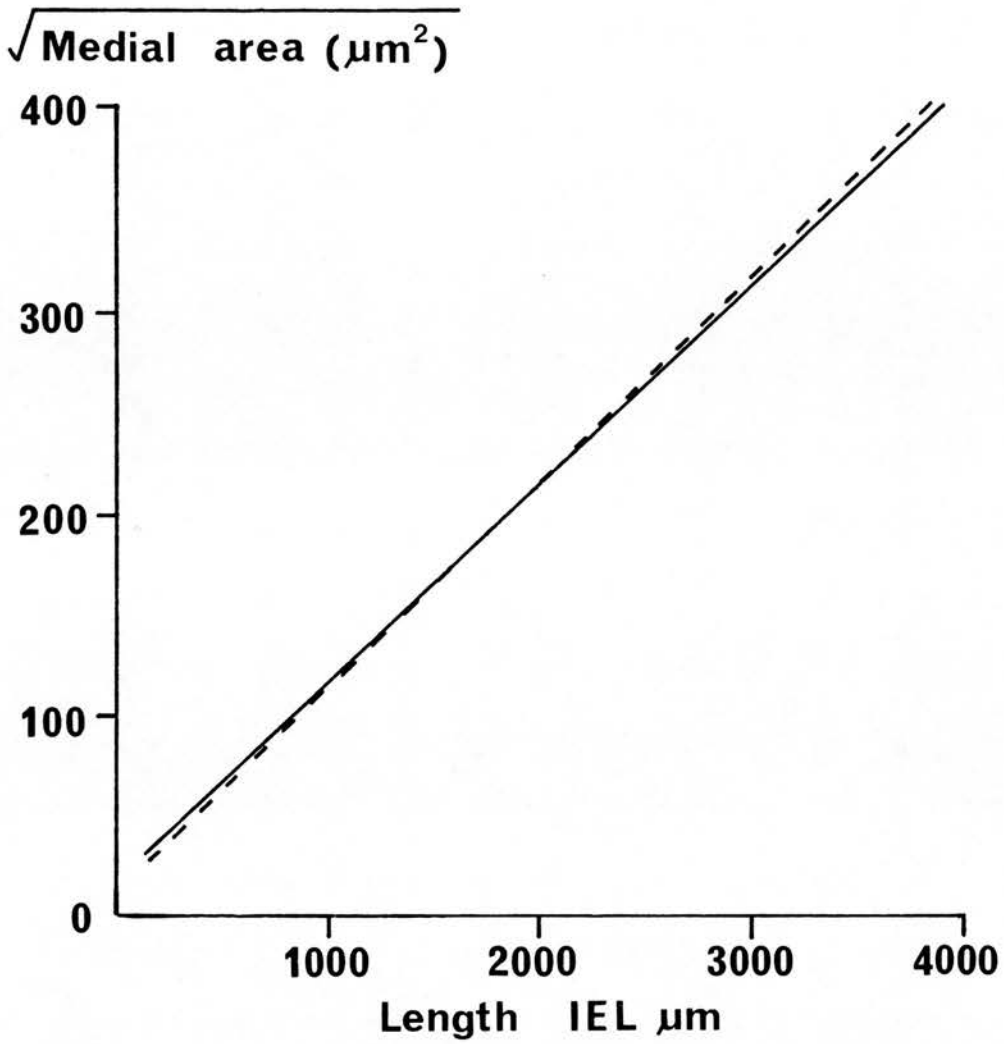


Figure 2.33 The relationship between square root of medial area and length of internal elastic lamina in the arteries of subject 10.

The lines of best fit are:

(———) $\sqrt{MA(1)}$ v IEL (1) $y = 20.00 + 0.10x$ $r = 0.99$

(- - -) $\sqrt{MA(2)}$ v estimated IEL (1) - crinkle grading method $y = 19.07 + 0.10x$ $r = 0.97$

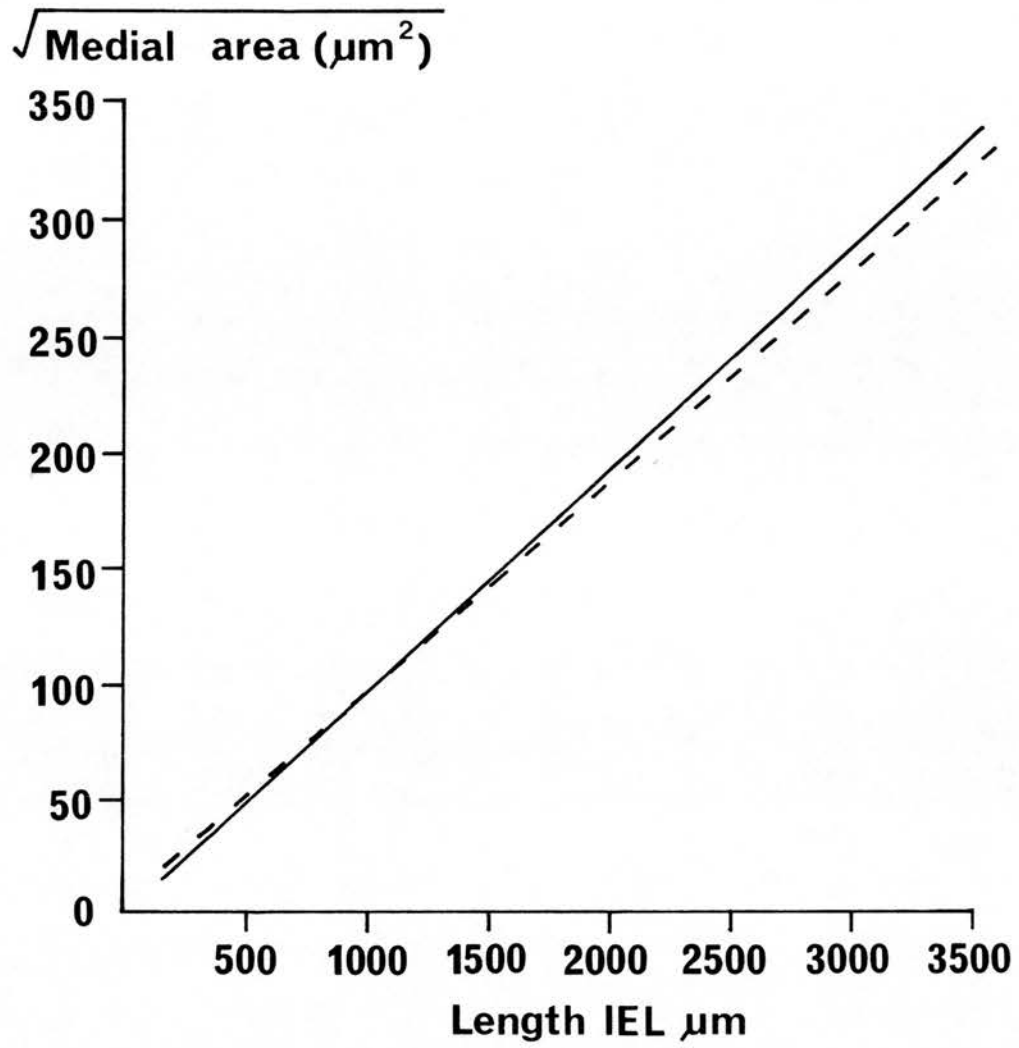


Figure 2.34 The relationship between square root of medial area and length of internal elastic lamina in the arteries of subject 11.

The lines of best fit are:

(———) $\sqrt{MA(1)}$ v IEL (1) y = 7.53 + 0.10x r = 0.98

(- - -) $\sqrt{MA(2)}$ v estimated IEL (1) - crinkle grading method y = 12.83 + 0.09x r = 0.97

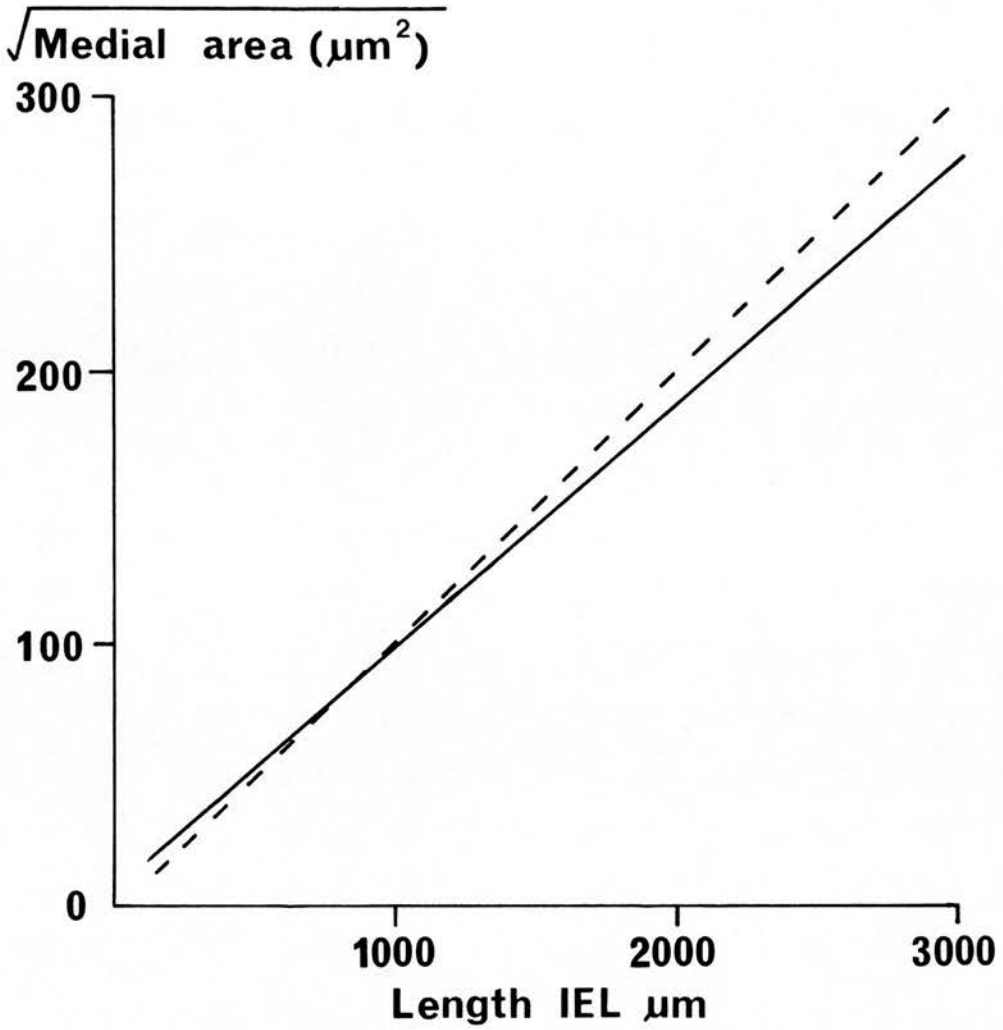


Figure 2.35 The relationship between square root of medial area and length of internal elastic lamina in the arteries of subject 12.

The lines of best fit are:

(———) $\sqrt{MA(1)}$ v IEL (1)	y = 7.78 + 0.09x	r = 0.99
(- - -) $\sqrt{MA(2)}$ v estimated IEL (1) - crinkle grading method	y = -2.65 + 0.10x	r = 0.98

Table 2.20 Mean (standard deviation) values of Intima Index (1) and (2) in the arteries of the three subjects, sub-divided by size.

	Measured or estimated length of internal elastic lamina					
	< 1000μm		1000 - 2000μm		> 2000μm	
Subject	*II (1)	II (2)	II (1)	II (2)	II (1)	II (2)
10	0.18 (0.09)	0.17 (0.07)	0.08 (0.03)	0.08 (0.03)	0.03 (0.02)	0.04 (0.03)
11	0.19 (0.08)	0.19 (0.09)	0.08 (0.04)	0.08 (0.04)	0.05 (0.02)	0.06 (0.006)
12	0.10 (0.03)	0.09 (0.03)	0.05 (0.02)	0.05 (0.01)	0.03 (0.006)	0.03 (0.005)

* II = Intima Index

2.4.20 Summary of Main Results and Conclusions

1. Using the digitiser the reproducibility of measurements of all parameters of muscular pulmonary arteries is excellent, with the exception of medial thickness.

2. It is essential to digitise at an appropriate magnification, e.g. one at which crinkles in the internal elastic lamina are clearly visible and easy to trace.

3. The criteria for an artery being considered 'digitisable' (cross-sectionally cut with an intact and well-defined internal elastic lamina round at least $^{7}/_{8}$ths of its wall) are adequately stringent.

4. The 'digitisable' muscular pulmonary arteries are representative of the total population.

5. The relationship between medial area and artery size (total length of internal elastic lamina) takes the form $y = Ax^{b}$; this is best linearised by plotting the square root of medial area against artery size.

6. Distension of pulmonary arteries with an injection medium causes the internal elastic lamina to stretch, by a factor of approximately 1.5.

7. Neither the pressure nor method of lung inflation/fixation affects the relationship between medial area and artery size as as described in 5.

8. Embedding tissue blocks in paraffin wax and sectioning causes considerable tissue shrinkage and compression; with glycol methacrylate the shrinkage and compression artefacts are minimal.

9. The shrinkage and compression of tissue samples in paraffin wax does not affect the relationship between medial area and artery size as described in 5.

10. More muscular pulmonary arteries are considered 'digitisable' in glycol methacrylate embedded tissue compared to paraffin embedded tissue.

11. There is no consistent relationship between area of intima and artery size for different subjects.

12. Similarly, there is no consistent relationship between Intima Index and artery size for different subjects.

13. The best way of expressing intimal abnormality in a subject, and comparing different subjects, is to calculate mean Intima Indices for muscular pulmonary arteries sub-divided by size.

14. It is possible to approximate the medial and intimal areas of cross-sectionally cut arteries not considered 'digitisable' by ignoring the crinkles in the elastic laminae and simply delineating the boundaries of the intima and media, and media and adventitia.

15. The best method of obtaining an estimate of the total length of the internal elastic lamina in cross-sectionally cut arteries not considered 'digitisable' is to multiply the length of the boundary between intima and media by a factor based on a by-eye estimate of the amount of collapse/constriction in that artery.

16. Muscular pulmonary arteries are not uniformly constricted/collapsed round the circumference of their walls; the overall degree of collapse or constriction appears to be affected by the size of the artery.

2.5 DISCUSSION

The lay-out of the discussion of this chapter is as follows. First of all there is a brief introductory section dealing with the advantages of measuring medial and intimal areas in the assessment of these two vascular components, and the advantages of defining artery size in terms of the length of the internal elastic lamina. Next, the Discussion concentrates quite specifically on the method chosen to measure the aforementioned parameters and what advantages/ disadvantages it is considered to have. The points raised are ordered in such a way that the Aims of the chapter (detailed in section 2.2) are dealt with in order; these points concern:-

1. The technique itself
2. Reproducibility of measurements obtained
3. Criteria for 'digitisibility'
4. Comparison of measured arteries wih total population
5. The relationship between medial area and artery size
6. The effect of different tissue preparation methods on pulmonary arteries
7. Assessment of patchy intimal abnormality
8. Maximising the data obtainable from pulmonary arteries

Where key results are referred to the appropriate section numbers are given in brackets.

2.5.1 Advantages and Disadvantages of the Various Parameters Used in the Assessment of Medial Hypertrophy, Intimal Abnormality and Artery Size

In the Introduction to this chapter there was a fairly comprehensive review of the parameters used by other workers to assess medial hypertrophy, intimal abnormality and artery size, and their methods of measuring them. The present section concerns itself with the parameters themselves and briefly discusses the advantages and disadvantages of each.

It is appropriate to start by emphasising once again the importance of choosing parameters that are unaffected by collapse or constriction of the artery. Failure to do so leads only to doubts and uncertainties concerning the results obtained. With respect to the media and intima of muscular pulmonary arteries each component may be assessed by measurement of either its thickness or area; these are the only two choices available. In uninjected arteries the former approach is fundamentally flawed but even in injected arteries thickness may not be a sensible indicator of either medial hypertrophy or intimal abnormality since it has been pointed out that the pulmonary arteries sometimes dilate excessively and unpredictably (Wagenvoort & Wagenvoort, 1977) and that changes in the intima may affect distensibility of an artery (Warnock & Kunzmann, 1977a). Area, therefore, would appear to be the most sensible parameter on which to base assessment of the medial and intimal components of pulmonary arteries. It is a measurement which is unaffected by post-mortem collapse or constriction and

furthermore in the case of the intima it is a measurement which is unaffected by any irregularity in the distribution of the intimal layer round the artery wall.

If one simply wants to determine whether or not medial hypertrophy has occurred in the pulmonary arteries of the lung as a whole then the technique of Wagenvoort (1960), namely expression of medial area per unit area of histological section, has much to recommend it. However, most workers who study the pulmonary vascular bed are interested in determining which size groups of arteries are most affected with respect to medial hypertrophy or intimal abnormality, and whether the lung is uniformly or patchily affected. This is not possible using Wagenvoort's technique. It is essential to have some indicator of artery size or some base-line parameter to which the measurements of the media and intima can be related. Ideally, this indicator should be unaffected by post-mortem collapse or constriction of arteries. There are several choices available, none of which is entirely satisfactory since it is impossible to know what the true size of any artery was during life. Nevertheless some sensible parameter must be chosen. Some workers have defined artery size by position relative to accompanying airway (e.g. O'Neal et al., 1955), others, specifically Naeye, have used the cross-sectional area of either the intima plus internal elastic lamina (e.g. Naeye, 1961a; Naeye, 1961b) or the area of the arterial intimal nuclei (e.g. Naeye, 1966; Naeye, 1969; Naeye & Dellinger, 1971) as a base-line to which medial area could be related. One of the major drawbacks of the technique of O'Neal et al. is that there is disparity between the branching patterns of the pulmonary artery

and bronchial tree, a finding which came to light in a study by Elliott & Reid (1965). With regard to Naeye's techniques their major flaw is that assessment of medial hypertrophy has to be limited to those arteries in which intimal change is absent. As changes in the intima of pulmonary arteries may be brought about by natural processes such as ageing (see Chapter 3) Naeye's method of assessment will exclude not only any diseased arteries but also some otherwise 'normal' arteries. The value of measurements obtained from such a highly selected group of arteries is questionable.

Only two 'size-specific' indicators of artery size are available, diameter or total length of internal elastic lamina. In uninjected arteries diameter is obviously an imprecise indicator of the true size of an artery. Unfortunately distension of arteries with an injection medium is not an ideal solution to this problem; the criticisms made earlier in this section with regard to the use of wall thickness measurements in injected arteries are equally applicable to diameter measurements. This leaves the total length of the internal elastic lamina. On theoretical grounds it is a good indicator of artery size. It is a measurement which is unaffected by collapse or constriction and measurements are potentially possible on all arteries satisfying certain criteria regarding angle of cut. Furthermore, the concept of theoretically 'unwrinkling' a vessel to determine its uncollapsed/unconstricted size is one which is readily understood. An added advantage of using this parameter is that all arteries are effectively reduced to the same state.

2.5.2 Advantages and Disadvantages of Using a Digitiser to Measure Muscular Pulmonary Arteries

Other workers have measured the medial area of pulmonary arteries but none have measured intimal areas specifically for assessment of the intimal component. Some of these workers (e.g. Furuyama, 1962; Honda, 1967; Kamal & Campbell, 1979) have also defined artey size in terms of the total length of the internal elastic lamina. In most instances medial areas have been determined either by calculation from micrometer measurements (not a precise method especially in uninjected arteries) or by planimetry (from tracings). In one of Weibel's studies (1970a) a point-counting method was employed, the measurements being obtained directly from histological sections.

In general, the methods chosen by other workers to measure the total length of the internal elastic lamina are extremely time consuming and tedious. The most common methods involve tracing an image of the artery produced either by projection (e.g. Niwa, 1971; Suwa & Takahashi, 1971; Yamaki & Wagenvoort, 1981) or a microscope 'camera lucida' (e.g. Cook & Yates, 1972a; Kamal & Campbell, 1979) and measuring the length of the internal elastic lamina on the tracing with a rotameter (e.g. Cook & Yates, 1972a) or by attaching a thin cotton thread to the line of it and measuring the length used (e.g. Honda, 1967; Kon, 1963; Yamaki & Tezuka, 1976). In Weibel's study (1970a) the length of the internal elastic lamina was obtained by stereologic counting methods.

At the outset of this study no one had used a method similar to the present one for measurement of the aforementioned parameters of pulmonary arteries. During the course of the study, however, there has been a report (Matsubara et al., 1984) of a histometrical investigation of pulmonary arteries in severe hepatic disease. In this particular study Matsubara et al. state that histological sections were traced on a screen for morphometry at appropriate magnifications; outlines of the internal and external elastic laminae were drawn with a 'light pen', their circumferences measured and the cross-sectional area of the media determined. The system used was an Image Analysing system obtained from Muto and Canon, Tokyo. Although no further details of the system are given it is obviously similar to the one described in the present study.

The advantages of using a digitiser for measuring pulmonary arteries are so considerable that it would have been surprising if reports had not begun to appear in which such a method was used. Perhaps the greatest advantage of a digitising system for measuring medial and intimal areas and total lengths of internal elastic laminae is that the measurements can be obtained directly from histological sections. This greatly reduces the work-load compared with other methods of measurement since tracings of the arteries are not required. In addition, tiresome calculations to account for magnification are unnecessary; this further simplifies the process of obtaining the required measurements. While it is true that these advantages are shared by Weibel's method of measurement, a digitising system has the edge on Weibel's technique in that the data are stored on tape and they can, by simple programming, be

handled in a variety of ways. It has also been pointed out (Glagov et al., 1981) that although point-counting is as accurate as contour tracing (digitising) for summing areas of large numbers of profiles, contour tracing is more accurate than point-counting for determining the area of an individual profile.

It seems that the only disadvantage of a digitising system for measuring pulmonary arteries is its cost. However, since this study was started the cost of such a system has greatly decreased. Furthermore the very flexibility of the system allows it to be used for measuring a variety of structures, not just pulmonary arteries, so in most research establishments it can be a multi-purpose measuring system and even used as a computer in its own right.

With regard to the measurement of pulmonary arteries this study has included only muscular pulmonary arteries since these arteries, particularly the smaller ones, form one of the most reactive parts of the pulmonary circulation. Using the digitiser it is, however, equally easy to measure the medial component of partially-muscular arteries or the intimal component of either partially-muscular or non-muscular arteries if so desired.

2.5.3 Reproducibility of Measurements Obtained Using the Digitiser

Right from the start of this study this was considered to be an area of immense importance for two main reasons. Firstly, a new technique was being developed which, it was hoped, would turn out to be an improvement on other currently used techniques; validation of the technique was, therefore, required. Secondly, regardless of

what technique is chosen for measuring pulmonary arteries, it is difficult to accurately compare quantitative changes in the medial and/or intimal components in disease states with the 'normal' if there has been no attempt, either to estimate the errors involved in measuring the components, or, for that matter, to investigate factors which might affect the measurements themselves.

It seemed sensible to carry out the reproducibility tests using muscular pulmonary arteries from subjects with a variety of cardio-pulmonary disorders. By so doing it would determine whether or not the technique could be widely applied to the assessment of medial hypertrophy and intimal abnormality. Also, any problem areas in the measurement of pulmonary arteries would be highlighted.

The initial assessment of observer error in the measurement of 'arteries' of known dimensions showed this particular error to be less than 2% (section 2.4.1), which was considered acceptable. In general, the reproducibility of the measurements of all parameters (intimal area is specifically discussed in section 2.5.8) of the 15 selected muscular pulmonary arteries was excellent (sections 2.4.3 (i) and (iv)) and did not vary much with the time interval between repeat measurements (section 2.4.3 (ii)). However, there was one notable exception and that was the parameter medial thickness. The very poor reproducibility of this measurement in uninjected arteries adds further criticism to that already levelled by Short (1962) at studies that have used this measurement, one which is greatly affected by post-mortem collapse or constriction, in the assessment of medial hypertrophy.

It was surprising to find that the reproducibility was equally poor in injected arteries, although comments of this nature have been made by Cook & Yates (1972a). Another observation of particular interest was that some arteries appeared to have resisted distension and showed quite marked crenation of the elastic laminae. These findings cast some doubt on the results obtained by the research groups headed by Lynne Reid, who inject the pulmonary arteries of their specimens, and assess medial hypertrophy by the thickness of the medial layer and artery size by diameter. Although measurements of the parameter external diameter were consistently reproducible using the present technique, expression of medial thickness as a percentage of vessel diameter does not reduce the inherent error in the medial thickness measurement, and conclusions drawn from innumerable such studies may well be suspect.

The extent to which constriction or post-mortem collapse can affect the percentage medial thickness measurement is particularly well illustrated by one of the 15 arteries included in the group used for reproducibility testing (Figure 2.36). The observed percentage medial thickness [Mean (M1 + M2 + M3 + M4)/Mean (D1 + D2) x 100] of this artery is 39.8% whereas its true percentage medial thickness (derived using the medial area and total length of internal elastic lamina measurements) is only 14.4%. Such findings raise serious doubts about the validity of the frequently used 'wall thickness' methods in the assessment of medial hypertrophy.

Measurements of medial area, unlike medial thickness, were generally very reproducible. Where evident, poorer reproducibility (greater than 5% variation) was linked to the structure of the

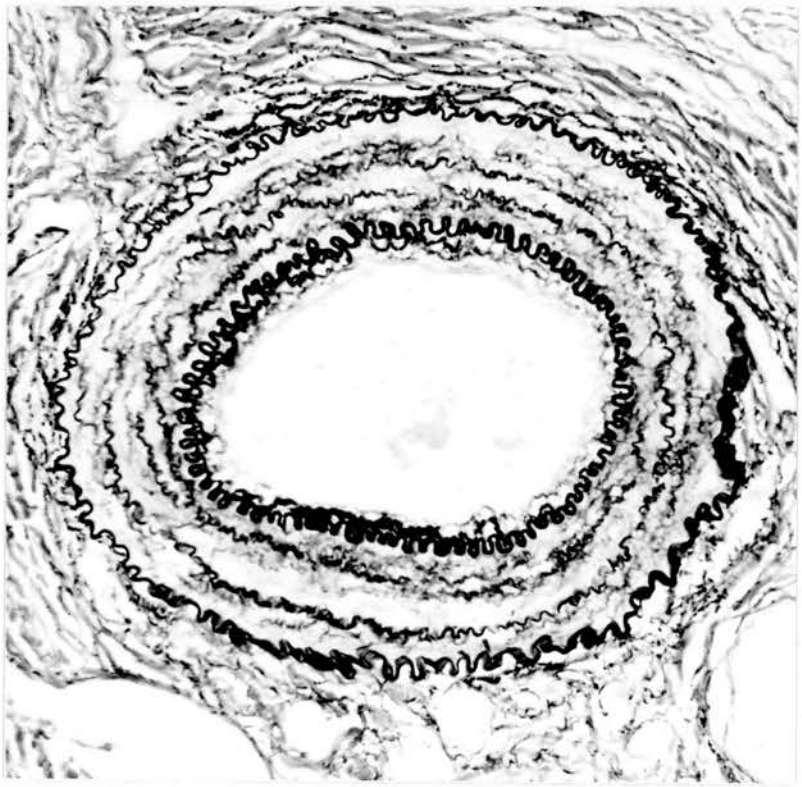


Figure 2.36 One of the muscular pulmonary arteries showing marked constriction.
x 180
Elastic Stain

artery, e.g. the very thin media of injected arteries or very small arteries. The former is of no real importance as the described technique is intended for use on uninjected material. With regard to the very small muscular pulmonary arteries it is encouraging that such arteries can be measured by the described technique as it makes it possible to accurately determine how far distally muscular arterial tissue extends. The level of reproducibility of the measurements of some of the parameters of these arteries may have been disappointing when compared with the larger arteries but this must be put in perspective. It is extremely difficult to accurately measure small areas, and in view of this a level of reproducibility within 10% is considered perfectly acceptable.

The investigation of the effect of magnification on the measurements obtained (section 2.4.3 (iii)) highlighted some problem areas with regard to the technique itself, and helped bring about a minor modification to the equipment used, reduction in the size of the point of light emitted from the electronic cursor, resulting in greater accuracy of the measurements obtained (section 2.4.3 (iv)). Some parameters were less affected by magnification than others; in general the diameter and area measurements were less affected than the length measurements, particularly the total length of the internal elastic lamina. This may seem a problem when artery size is defined in terms of the total length of the internal elastic lamina. However, even in very constricted or collapsed arteries it is possible to obtain an accurate measurement for this parameter providing that the correct magnification is chosen, one at which the crinkles in the lamina are clearly defined. The group of arteries

most affected by this problem are the larger ones which have to be viewed at low magnification. Fortuntely, measurements are just as easily obtained from photographs using the digitiser, so the solution to this problem is straightforward.

Using a reduced light source on the electronic cursor and viewing arteries at an appropriate magnification the described technique will produce measurements of muscular pulmonary arteries that are accurate and reproducible to within 5% in most instances. It is impossible to discuss how this compares with other techniques used for measuring, for example, medial area and total length of internal elastic lamina. The vast majority of researchers have neglected to mention reproducibility testing in their published papers, probably because it is an area they have not investigated. Exceptions are Hunter et al. (1974) and Hale et al. (1980) but their techniques for assessing the media and size of pulmonary arteries are fundamentally different to the one described here. None of the studies using techniques similar in principle to this one have mentioned reproducibility of the measurements obtained. Cook & Yates who measured the length of the internal lamina from tracings using a rotameter state -

> "After skill with the instrument had been gained, the magnification at which arteries were drawn permitted satisfactory negotiation with the rotameter of the curves of the lamina of collapsed renal arteries" Cook & Yates (1972a).

These workers are stating that a 'learning period' is necessary while an operator becomes familiar with the method of measuring the length of the internal elastic lamina. This is true regardless of the technique used and was certainly the case in the present study,

in which all the reproducibility testing was carried out after experience with the method of measurement had been gained. However, Cook & Yates, by omission, are implying that once experience has been gained there are no other factors which might affect the measurements obtained. As the present study has shown this is certainly not the case.

It remains to be seen what the inter-observer variation is in the measurement of pulmonary arteries using the digitiser. However, the technique is simple and, as pointed out in the following section, the criteria for an artery being considered 'digitisable' (measurable using Program 1) are stringent; consequently the inter-observer variation is not expected to be much higher than the intra-observer variation described.

2.5.4 Criteria for 'Digitisibility'

In this study reproducibility testing was carried one step further and applied to the selection of muscular pulmonary arteries considered 'digitisable'. Criteria for an artery being considered 'digitisable' were defined and subsequently tested; these criteria were found to be stringent and unaffected by the severity of disease present (section 2.4.4). Only cross-sectionally cut arteries were measured and then only if they had an intact and well-defined internal elastic lamina round at least $^{7}/_{8}$ths of their walls. Some workers, e.g. Hasleton *et al.* (1968), Heath & Best (1958) and Hicken *et al.* (1965) have insisted, not only that arteries be cross-sectionally cut, but also that they be circular. In the author's experience circular arteries are rarely seen. This has also been

remarked upon by Suwa & Takahashi (1971). They considered that so long as an artery was cross-sectionally cut, as determined by the appearance of the internal elastic lamina (no 'smearing') and the orientation of the smooth muscle fibres, circularity of the arteries was a non-essential criterion. In addition to the two criteria applied by Suwa & Takahashi, obliquely cut arteries were recognised in the present study by an increase in wall thickness at two opposite points on one particular diameter.

Apart from the angle of cut and the state of the internal elastic lamina no further exclusion criteria were applied in the selection of arteries for measurement. As mentioned in the Introduction to this chapter, the commonest exclusion criteria applied by other workers are related to size of artery or, specifically in the assessment of medial hypertrophy, to the presence of severe intimal abnormality which is thought to cause secondary thinning of the media (Wagenvoort & Wagenvoort, 1982a). In the present study all sizes of muscular pulmonary arteries were measured both with regard to assessment of the media (see section 2.5.6 in particular) and the intima (see section 2.5.8). Furthermore, in the assessment of the media even arteries with severe intimal abnormality were included. Although it may very well be true that severe intimal abnormality brings about medial atrophy this has still to be proved using a reliable measuring technique.

2.5.5 'Digitisable' Arteries: How They Compare with the Total Muscular Pulmonary Artery Population

One of the drawbacks of defining artery size in terms of the total length of the internal elastic lamina is that only arteries with an intact and well-defined internal elastic lamina can be measured. The measuring technique described in this study is, therefore, of no practical use in the assessment of medial hypertrophy or intimal abnormality in cases or disease states where there is fragmentation or destruction of the internal elastic lamina. Fortunately this occurs infrequently, and certainly not in association with the most common cardio-pulmonary disorders, such as hypoxic pulmonary vascular disease on which interest is centred. Nevertheless, it is worth bearing in mind that in view of the criteria imposed only a proportion of arteries will be measured using techniques such as the present one. This is in marked contrast to the 'wall thickness' methods of measurement in which, as an absolute minimum, all cross-sectionally cut arteries can be measured. However, the present study has shown that the arteries selected for measurement, although few in number, are a representative sample of the total muscular pulmonary artery population, at least in terms of muscle or intimal thickness expressed as a percentage of artery diameter (section 2.4.5). It was somewhat unfortunate that this particular aspect of the study had to be based on a measurement showing poor reproducibility (sections 2.4.3 (i) and (iii)) but this was considered justifiable on the grounds that no other measure of the media or intima was possible on all arteries. Other workers using length of internal elastic lamina as

an indicator of artery size have not attempted to compare arteries selected for measurement with the total population yet, as with reproducibility testing, this is really an area of considerable importance in the validation of such a technique.

With regard to the intima specifically, it has to be admitted that there were doubts as to whether arteries considered 'digitisable' would, in the majority of cases, be a representative sample of the total population, even in view of the convincing results obtained for the four subjects studied. These subjects, in retrospect, were perhaps an unsuitable group in that the intimal changes were quite severe and evenly distributed, not only round the walls of individual arteries, but also in arteries throughout the lung. In most disease states intimal changes do not affect the entire lung uniformly nor are they evenly distributed round the artery wall (Harris & Heath, 1977; Wagenvoort & Wagenvoort, 1977). These doubts turned out to be less important than first imagined in that a technique was developed which allowed medial and intimal area measurements to be obtained for those cross-sectionally cut arteries with an ill-defined internal elastic lamina (see section 2.5.9), thereby effectively increasing the number of arteries measured.

2.5.6 The Relationship between Medial Area and Artery Size

In determining the relationship between the amount of muscle in the wall of a muscular pulmonary artery and its size it was decided to use the measurements medial area and total length of internal elastic lamina *per se*. Most other workers (e.g. Cook & Yates,

1972a; Furuyama, 1962; Niwa, 1971; Yamaki & Horiuchi, 1979) who have measured these two parameters in either pulmonary or systemic arteries have converted the measurements to those of wall thickness and radius/diameter. Although this practice is theoretically sound, and produces values that are more readily visualized than medial area and total length of internal elastic lamina, it was felt that it helps perpetuate the direct 'wall thickness' methods of measurement, which are inaccurate, and for this reason it was avoided. It is interesting that the vast majority of the aforementioned workers (e.g. Furuyama, 1962; Niwa, 1971; Yamaki & Horiuchi, 1979) have chosen to define the anatomical diameter of an artery as lying between the mid-points of the media on opposite sides of the artery. Cook & Yates could see no value in this approach and state -

> "Surely it is better that one's estimate of an artery's diameter should remain constant rather than alter if the vessel undergoes medial hypertrophy?" Cook & Yates (1972a)

The author is in complete agreement with this statement.

The relationship which exists between medial area (or derived medial thickness) and total length of internal elastic lamina (or derived radius/diameter) in a vascular system composed of tapering tubes takes the form $y = Ax^b$ (section 2.4.6), which creates problems when sets of data are to be compared. The first step towards solving the problem is to convert the relationship to a linear one. In this study it was tried plotting the data using single or double logarithmic coordinate systems as other workers have done, e.g. Weibel (1970a), or Honda (1967), Suwa & Takahashi (1971) and Yamaki & Tezuka (1976) respectively. Using the single logarithmic system it was found that the function describing the best fit between the

two variables took the form $y = Ax^b$. In Weibel's study (1970a) it is obvious from the graph shown that the relationship between log. medial area and circumference of internal elastic lamina, as he calls it, is not linear but takes the form $y = Ax^b$. Some of those workers electing to use double logarithmic coordinate systems, taking Furuyama as an example, found that the regression line between log. medial thickness and log. anatomical radius was deflected in the region of arteries with an anatomical radius of 100μm (Furuyama, 1962). This may or may not be the case, but looking at the graphs shown (Furuyama, 1962) 100μm seems to be a rather arbitrary cut-off point. However, the important point emerging from studies such as Furuyama's is that the best fit between measures of the medial component and size of arteries plotted on a double logarithmic system is not, overall, a linear one. This was found in the present study (section 2.4.6). To get an overall best fit that was linear required that the square root of the medial area to be plotted against the length of the internal elastic lamina. To the author's knowledge this method of expressing the data has not been used previously. Having linearised the relationship between medial area and artery size any two sets of data could then be compared by testing the hypothesis that a common slope existed between the two regression lines, using standard statistical procedures. This approach was used to assess the effect of different tissue preparation methods on the measurements obtained from muscular pulmonary arteries, which is discussed in the following section.

2.5.7 The Effect of Different Tissue Preparation Methods on Measurements of Pulmonary Arteries

The factors investigated were:-

1. Arterial distension with an injection medium
2. Different methods/pressures of lung inflation/fixation
3. Different tissue embedding media

In general these factors were not of interest in themselves, the purpose of investigating their effect was mainly to determine how strictly controlled the tissue preparation methods needed to be when studying the pulmonary vasculature. Arterial distension was an exception; so many studies have been carried out on pulmonary arteries distended by an injection medium that it was considered important to quantitate the effect of this procedure. As the vast majority of these studies, notably those of Lynne Reid and colleagues, have used hypertensive pressures this practice was copied in the present study.

The relationship between medial area (square root of) and total length of internal elastic lamina was significantly different for injected and uninjected pulmonary arteries as determined by the slopes of the regression lines. These differences were such that for a given medial area the internal elastic lamina was longer by a factor of approximately 1.5 in the injected arteries (section 2.4.7). This is well within the value of 2.5 which Reuterwall as quoted by Burton (1954) states is the point at which elastic fibres begin to break.

Several other workers have looked at the effect of arterial distension on measurements of the media and size of pulmonary arteries. Using physiological pressures Wagenvoort (1960) found little difference in the mean percentage wall thickness of injected and uninjected muscular pulmonary arteries, which implies that at these pressures the internal elastic lamina does not stretch, a finding reported by Cook et al. (1975). In contrast, Kamal & Campbell (1979), studying the small intestinal arteries in systemic hypertension, found that injection at the systolic pressures recorded during life caused the internal elastic lamina to stretch. These discrepancies are likely to be related to different measuring techniques, and also to differences in the injection media used in these three studies. Kamal & Campbell do not state how much of an increase there was in the length of the internal elastic lamina but calculation from the data recorded on their graphs reveals the factor to be less than the 1.5 found in the present study. This difference is probably explained by the fact that the injection pressures used in the present study were markedly hypertensive for the pulmonary circulation.

In their reported study Kamal & Campbell make a very important point which is perhaps pertinent to discuss here. When discussing a study by Cook & Yates (1972b) they state -

> "The belief that the vessels were fully distended if the internal elastic lamina was rendered smooth and free from convolutions may be fallacious. The architecture of the lamina, a felt-like or close-meshed plexus (Dees, 1923), could allow for changes in the calibre of the vessel within the limit of its appearance, in cross-section, as a smooth non-undulating membrane" Kamal & Campbell (1979).

Most workers who inject the pulmonary or systemic arteries are in fact assuming that all arteries are being reduced to the same state (completely distended) simply because the lamina is unwrinkled. That this is not so is yet another serious flaw in the practice of obtaining measurements from injected arteries. This comment of Kamal & Campbell's ties in with a point made by Warnock & Kunzmann (1977a), which was that the distensibility of pulmonary arteries may be reduced as the degree of intimal thickening increases. Unfortunately the present study cannot confirm or refute this statement as all four subjects studied were broadly similar in terms of degree of intimal change (which was quite severe) and in their distensibility factors. It may be that the stretching factor of 1.5 observed in the present study relates only to subjects with that particular degree of intimal abnormality, and is completely different in subjects with no intimal abnormality. This is an interesting point and one which is certainly worthy of further investigation.

With regard to lung <u>inflation/fixation</u> (which was done intrabronchially), it was encouraging to find that the described method of measuring pulmonary arteries using a digitiser is possible on uninflated material (section 2.4.8) as this makes it of potential use in the evaluation of vascular changes in biopsy specimens. Also encouraging was the finding that the overall relationship between medial area and size of artery is unaffected by the pressure or method used to inflate a lung (section 2.4.9), which it was hoped would be the case. This is of considerable practical importance as

it dispenses with the need for complicated pressure controlled lung inflation apparatus and, in addition, it allows a direct comparison of measurements of pulmonary arteries from lungs which have been inflated to differing degrees.

It was rather surprising that the difference between arteries from uninflated and routinely inflated lungs were slight and not significant. On the assumption that the volume of muscle present would be the same but that the length of the arterial pathway would be shorter in the uninflated lung, it had been expected that more muscle would be present in cross-sectionally cut arteries from the uninflated lung. It is, however, difficult to imagine how the internal elastic lamina behaves in such a situation, and the lack of a significant difference in the overall relationship between medial area and artery size may well be related to this unknown factor.

Other workers in the field of pulmonary arteries have made no attempt to quantitate the effect of different tissue fixation procedures on the measurements obtained by their techniques. While it is true that there is no effect, this applies only when medial area is measured and artery size defined in terms of the total length of the internal elastic lamina. The statement may be untrue in cases where the method of measurement is based on the 'wall thickness' concept. If so, then the results of studies such as that of Wagenvoort & Wagenvoort (1970), based on material obtained from a variety of sources and prepared in a variety of ways, may be invalid.

A number of workers, e.g. Fukaya & Martin (1969), Tsunoda & Martin (1973) and Weibel & Vidone (1961) have carried out very detailed studies to determine the correction factors that need to be applied to account for the artificial shrinkage of lung tissue during fixation and paraffin embedding and sectioning. Of these workers Weibel & Vidone (1961) found that the greatest shrinkage occurred during the fixation process. This is contrary to the findings of the other two groups and undoubtedly related to the formalin steam method applied by Weibel & Vidone for tissue fixation. The consensus of opinion is that most of the tissue shrinkage occurs as a result of paraffin embedding.

Few workers quantitating either the medial or intimal component of pulmonary arteries have bothered to apply correction factors to the measurements obtained; exceptions are Hale et al. (1980). In the present study the purpose of investigating tissue shrinkage and compression during paraffin embedding and sectioning was not to add yet another series of correction factors to those already published. All other studies of the pulmonary vasculature have embedded tissue in paraffin; the present study wished to explore the advantages and disadvantages of using glycol methacrylate instead, and also to quantitate any differences in the effect of these two tissue embedding media on the measurements obtained from muscular pulmonary arteries. As expected the shrinkage that occurred during paraffin embedding was considerable, the percentage linear and area reductions in sample size being 16% and 29% respectively (section 2.4.10); these values are somewhat higher than the correction factors published by Fukaya & Martin (1969), Tsunoda & Martin (1973)

and Weibel & Vidone (1961), which range from 1.09 to 1.10 (linear) and 1.18 to 1.21 (area). The values are nearer to the 33% shrinkage reported by Baker (1960). Tsunoda & Martin (1973) also quote correction factors for the shrinkage of lung tissue during sectioning and staining, which was small, only 0.3 percent of the linear dimension. The present study has indicated that these 'compression' factors are much higher amounting, on average, to 7% of the linear dimension (section 2.4.10).

The biggest advantage of embedding tissue in glycol methacrylate is that virtually no tissue shrinkage occurs either during the embedding or sectioning process (section 2.4.10). A further advantage, and one which has long been realised (Ashley & Feder, 1966) is that with glycol methacrylate embedding the histological detail is much improved as is the overall quality of the sections produced. This is borne out by the increased number of muscular pulmonary arteries considered 'digitisable' in sections from glycol methacrylate embedded tissue compared to paraffin embedded tissue; in some cases this increase was more than threefold (section 2.4.11). For these reasons glycol methacrylate embedding is to be recommended to those engaged in quantitative studies of the pulmonary vasculature. Its only disadvantages are its cost, and the somewhat disappointing results obtained when taking photographs of elastic stained tissue, a finding also commented upon by Edwards et al. (1979).

Although shrinkage of tissue samples was considerable with paraffin embedding and sectioning, it was interesting that the relationship between medial area and length of internal elastic

lamina was essentially the same for arteries from glycol methacrylate or paraffin embedded tissue (section 2.4.11). This suggests that muscle and elastic tissue shrink by equivalent amounts during paraffin embedding. It is fortunate that this is so because it means that arteries from paraffin or glycol methacrylate embedded tissue can be directly compared, at least in terms of the slope of the regression line between medial area (square root of) and total length of internal elastic lamina.

2.5.8 Assessment of Intimal Abnormality

Quantitation of the intimal component of muscular pulmonary arteries, as with the medial component, comprises two somewhat separate stages; firstly, there is the measurement of the intima in the individual artery, and secondly, there is the analysis of that data.

With regard to methods of measuring the intima itself it is considered that the technique described in the present study is, for three main reasons, a considerable improvement on the rather crude 'intimal thickness' methods which were described in section 2.1.3 (iv) of the Introduction. Perhaps the most important improvement is that the present technique produces a measurement for the area of the intima and as such it is entirely unaffected by the distribution of the intimal layer round the wall of the artery. This is in marked contrast to the intimal thickness measurement (to date the most frequently used measurement in the assessment of the intima) which may under- or over-estimate the intimal layer if it is

irregular, eccentric or crescent-shaped as frequently happens in disease states (Harris & Heath, 1977; Wagenvoort & Wagenvoort, 1977).

The second reason concerns the parameter used to describe artery size and to which measurements of intimal area are related; the total length of the internal elastic lamina is a parameter which is unaffected by collapse or constriction of the artery. Other workers have chosen the parameter diameter, either internal (e.g. Wagenvoort & Wagenvoort, 1965a; Wagenvoort & Wagenvoort, 1970; Wagenvoort & Wagenvoort, 1973) or external (e.g. Hale et al., 1980), a measurement which varies considerably with the degree of collapse or constriction present. Expression of intimal thickness in relation to diameter leads to an over-estimation of the intimal component.

Thirdly, there is the reproducibility of the measurements of intimal area obtained using the digitiser, which, as a rule, varied less than 5% on subsequent digitisations (section 2.4.12 (ii)). Poorer reproducibility (up to 12% deviation) was evident only in arteries in which the intimal area was very small. Magnification did not appear to have much effect on the measurements of intimal area with the exception that digitisation at a lens objective magnification of x4 tended to produce under- or over-estimation of the intimal area (section 2.4.12 (iii)). The obvious solution to this problem is to digitise at lens objective magnifications of x10 upwards.

Although the described method of measurement will accurately assess the intimal component of individual arteries there are problems in handling the data and deciding how best to compare subjects. Regression of intimal area against artery size for the six study subjects, specifically chosen because they covered a variety of disease states, revealed that no single function will describe the relationship between these two parameters for all subjects (section 2.4.13). This is in marked contrast to the relationship between medial area and artery size which generally takes the form of $y = Ax^b$ (section 2.4.6). Since there is no consistent relationship between intimal area and artery size it is not possible to compare subjects or disease states simply by comparing the slopes of the regression lines, which is possible with the media.

One disadvantage of relating area of intima to artery size is that it is not immediately obvious from the graphs which arteries are most affected by intimal change, in relative terms. For this reason it was decided to express the data in another form, the Intima Index, in which the intimal area is expressed as a ratio of the area enclosed by the internal elastic lamina in its theoretically unwrinkled state. Theoretical, rather than actual, state was chosen to overcome the problems associated with variable constriction or post-mortem collapse of arteries. It was felt that the Intima Index gave a readily understandable impression of the extent of intimal abnormality in an artery as it ranges from >0 to $\leqslant 1$, indicating minimal through to total occlusion of the artery lumen. The relationship between the Intima Index of an artery and

the functional changes within it is not a simple one. Assuming a constant blood flow, the pressure in an artery with an Intima Index of, for example, 0.5 (i.e. a minimum effective reduction in lumen area of 50%) would not simply be increased by a minimum of 50%. Since pressure is inversely proportional to the magnitude of the fourth power of the radius (Poiseuille's Law) the pressure in such an artery would be increased by a factor of four at least. On the subject of lumen occlusion, the Intima Index described in the present study is similar to a method used for examining the coronary arteries, which was presented as a poster demonstration at a recent meeting of the Pathological Society of Great Britain and Ireland by Thomas & Davies (1984). They measured the degree of stenosis in coronary arteries, following injection with a gelatin/barium sulphate mixture, by comparing the area of the lumen with the area within the internal elastic lamina. This method, although similar in principle, is probably not as accurate as the present one due to the difficulties involved in choosing the distension pressure and trying to distend all arteries to the same extent.

An advantage of relating Intima Index, rather than intimal area, to artery size was the ease with which those arteries most affected by intimal change could be determined. Unfortunately no single function described the relationship between Intima Index and artery size for all subjects (section 2.4.14) making it impossible to compare subjects by any method other than the calculation of a mean Intima Index for arteries sub-divided by size (total length of internal elastic lamina). Some workers (e.g. Hale et al., 1980; Wagenvoort & Wagenvoort, 1965a) have simply calculated a mean value

for their measurements of the intima based on all arteries measured; it is, however, essential to subdivide by size as the smaller arteries are more affected by intimal change.

Finally, in this section on quantitation of intimal abnormality, it is appropriate to make some comment on the muscular pulmonary artery population selected for measurement. With the described method of measurement the number of arteries measured was less than 35 per subject with the paraffin embedded tissue (tissue from five of the six subjects studied was paraffin embedded). Taking into account the patchiness of intimal abnormality in the majority of subjects studied, the question is whether these 35 or fewer muscular pulmonary arteries were a truly representative sample of the total population. It is possible to compare the measured arteries with the total population; this was done for a group of four subjects earlier on in the study (section 2.4.5). However, such a comparison has to be based on the parameters intimal thickness and artery diameter, and it is of questionable value in view of the generally irregular distribution of the intimal layer round the artery wall, coupled with a variable degree of post-mortem collapse or constriction. The ideal solution to this possible problem of a non-representative artery population being selected for assessment of intimal abnormality is to increase the number of arteries measured. Embedding tissue in glycol methacrylate is helpful, e.g. for the one subject in this particular aspect of the study whose tissue was embedded in glycol methacrylate 96 arteries were considered 'digitisble'. Another possibility is to obtain a measurement for intimal area and length of internal elastic lamina

in those arteries which are cut in cross-section but not considered 'digitisable' using the standard Program 1 procedure. This is in fact possible using a simple adaptation of the measuring procedure used in conjunction with Program 1. The advantages and disadvantages of this abridged technique are discussed in the following section.

2.5.9 Maximising the Data Obtainable from Pulmonary Arteries

This study has established that it is possible to obtain measurements of medial and intimal area for muscular pulmonary arteries which, although cut in cross-section, have an ill-defined internal elastic lamina. More important it is also possible to approximate the total length of the internal elastic lamina in these arteries, providing that some muscular pulmonary arteries with well-defined internal elastic lamina have been measured. The technique used to obtain these measurements is basically an abridged version of the one used for measuring cross-sectional arteries with a well-defined internal elastic lamina.

In order to determine whether the abridged technique would work it had to be tested using pulmonary arteries for which the true values of medial and intimal area and total length of internal elastic lamina were known. As predicted from the outset there were no problems in approximating medial and intimal areas (section 2.4.16).

With regard to methods of estimating the length of an internal elastic lamina, two of the methods it was thought might work did

not, and the reasons for this in themselves reveal some interesting facts about constriction and post-mortem collapse in pulmonary arteries. Firstly, the degree of constriction/collapse is not uniform round an arterial wall (section 2.4.17 (ii)); consequently it is not possible to estimate the total length of an internal elastic lamina by multiplying the length of the boundary between intima and media by a 'crinkle factor' derived from part of that lamina. Secondly, the overall degree of crinkliness in an internal elastic lamina appears to be dependent upon artery size, larger arteries being more constricted/collapsed. This was evident in all three subjects studied but to varying extents (sections 2.4.17 (iii)). It remains to be seen whether this is a universal feature of muscular pulmonary arteries but, if so, then it is yet another reason for not estimating medial hypertrophy or intimal thickening in uninjected arteries by the commonly used 'wall thickness' methods. Not only will the measurements obtained vary with constriction, but arteries of different sizes will be unequally affected.

The remaining method of estimating the length of an internal elastic lamina, the simple by-eye crinkle grading method, produced what were considered to be acceptable estimates (section 2.4.17 (i)). Determination of the relationships between both medial area and intimal area (expressed as Intima Index) and artery size, first of all using the true values and then the estimated values, revealed that both sets of relationships were virtually identical (sections 2.4.18 and 2.4.19). Thus, the method of estimating medial and intimal areas was validated as was the method of estimating the

length of an internal elastic lamina. It should be pointed out that when grading arteries for degree of constriction/collapse there may well be variations between subjects (and within a subject) in arteries assigned a similar crinkle grade; this was certainly the case in the three subjects studied. These variations may be explained by differences in the depth of the crinkles and their distance apart.

There is only one major disadvantage of the described method for measuring cross-sectional arteries with an ill-defined internal elastic lamina and that is the increased work entailed in measuring those arteries with a well-defined internal elastic lamina; they have to be measured using both the standard and abridged techniques. However, this disadvantage of the technique is far outweighed by its advantages. The technique makes it possible to obtain medial and intimal area measurements for all arteries cut in cross-section, providing a proportion have a well-defined internal elastic lamina. This is of considerable practical importance especially with regard to the intima where it is considered advisable to maximise the data obtained in order to overcome the problems associated with an irregular distribution of intimal abnormality and a possible bias in the artery population selected for measurement using the standard technique. Measurement of all cross-sectionally cut arteries puts the technique on a par with most of the 'wall thickness' methods described in the Introduction, in terms of the number of arteries measured. In contrast to these techniques, however, the measurements of the media and intima and artery size (total length of

internal elastic lamina) have the advantage of being unaffected by constriction or post-mortem collapse of vessels.

CHAPTER 3

THE EFFECTS OF AGE AND SMOKING ON THE PULMONARY VASCULATURE

Chapter 3 comprises six main sections - an Introduction followed by a section detailing the Aims of the chapter, a Material and Methods, Results, Results Appendix, and finally a Discussion. The theme of this chapter is the effects of age and smoking on vessels in the pulmonary circulation, with special reference to the muscular pulmonary arteries.

3.1 INTRODUCTION

Workers who study the pulmonary vascular bed are interested in determining what structural alterations age and smoking bring about in its component vessels, particularly the muscular pulmonary arteries which form its most reactive part by virtue of their role in regulating pulmonary vascular resistance. The reasons for this interest are twofold. Coupled with a genuine interest in the effects of age and smoking there is the need to establish 'baseline' values so that structural changes in disease states may be accurately quantitated.

The purpose of this Introduction is to describe the reported effects of age and smoking on vessels in the pulmonary circulation dealing briefly with alterations in the:

- distribution of types of vessel by size
- composition of vascular walls
- physical and physiological characteristics of vessels.

These three sections are followed by two more detailed sections specifically centred on alterations in the medial and intimal components of pulmonary blood vessels. Finally, there is a section commenting on the measuring techniques employed in the assessment of the effects of age and smoking; the Aims of the chapter follow on from this.

3.1.1 Alterations in the Distribution of Vessel Types by Size

Dealing solely with the effects of ageing it has been reported (Ferencz *et al.*, 1967) that at any age pulmonary arteries of different histological/structural types, that is elastic, transitional, muscular and non-muscular, cover a limited range in terms of size; in undiseased persons the differences observed between birth and adult state with regard to the above size distributions were slight. This is at variance with the findings of Semmens (1970) who, in a study of injected pulmonary arteries of old individuals (age range 67 - 76) found differences in the size distributions of elastic, transitional and muscular arteries when compared with those found in young individuals by Elliott (1964) using identical methods. In the aged lungs the change from one type to another occurred at a larger diameter, implying loss of elastic tissue with age. This subject is further pursued in the next section.

3.1.2 Alterations in the Composition of Vascular Walls

In a study of the effects of age and smoking on small muscular pulmonary arteries (less than 150μm in diameter) Naeye & Dellinger (1971) measured the relative proportions of circularly orientated smooth muscle, longitudinally orientated smooth muscle, and collagen plus elastic tissue. They found that the percentage of pulmonary arterial walls comprised of normal circularly orientated smooth muscle decreased with increasing age, this at a faster rate in smokers than non-smokers. In contrast the proportion of collagen

increased with increasing age, smokers having a greater proportion in their arterial walls than non-smokers.

Also on the subject of collagen content it has been reported (Mackay et al., 1978) that the medial collagen content of the major arterial and venous branches at the lung hilum decreases with increasing age, results of chemical analysis confirming those obtained from morphological analysis by staining. So, either the results of the studies of Naeye & Dellinger (1971) and Mackay et al. (1978) are at odds with each other, a possibility due to differences in the methods used, or arteries of different structural types behave differently.

Naeye & Dellinger (1971) do not comment specifically upon the elastic tissue in the media of small muscular pulmonary arteries. Other workers (Harris et al., 1965) studying the pulmonary trunk have found a decrease in the amount of elastic tissue in the media with increasing age. It would appear that there are also alterations in the appearance of elastic fibres within the media, and in the elastic laminae themselves in young and old individuals. In muscular pulmonary arteries the elastic laminae are often coarse and irregular in older individuals (Wagenvoort & Wagenvoort, 1965a) and in the pulmonary veins increasing age tends to result in splitting and disappearance of elastic laminae and elastic fibres within the media (Wagenvoort & Wagenvoort, 1979).

Finally, in this section it is appropriate to comment upon the association between age and smoking habit and the appearance of longitudinally orientated smooth muscle cells within pulmonary

vascular walls, an area of considerable controversy. The occurrence of these cells has been noted in the intima of muscular pulmonary arteries by Naeye & Dellinger (1971) who found their incidence to be higher in smokers than non-smokers. These findings have been disputed by Semmens (1970) and Wagenvoort & Wagenvoort (1979) who, in studies of the effect of age on pulmonary arteries, could find no evidence of longitudinally orientated smooth muscle in aged non-smokers.

3.1.3 Alterations in the Physical/Physiological Characteristics of Pulmonary Vessels

As Wagenvoort rightly points out (Wagenvoort & Wagenvoort, 1977), the structural composition of pulmonary vascular walls is extremely important in determining their mechanical properties. It is not surprising, therefore, to find that the alterations in the make-up of vascular walls brought about by age and smoking may lead to functional alterations in the pulmonary circulation.

One feature of ageing which has been commented upon by a number of workers is the reduced extensibility or elasticity of vessels; this has been found in the pulmonary trunk (Harris et al., 1965), the main pulmonary artery (Banks et al., 1978), the major arterial and venous branches at the lung hilum (Mackay et al., 1978), and also in the pulmonary veins (Wagenvoort & Wagenvoort, 1979). Ageing is also said to result in a weakening of arterial wall tissue at all sites (Learoyd & Taylor, 1966). The reduced extensibility or elasticity of vessels has been attributed to an increase in the collagen content of the vascular wall (Harris et al., 1965).

However, Mackay et al. (1978), who claim that the medial collagen content of the major pulmonary arteries and veins decreases with age, are of the opinion that the reduced extensibility or increased stiffness of the wall results from changes in the elastic tissue at a molecular and lamellar level and has nothing whatsoever to do with the collagen content of the vascular wall. This loss of elasticity with age is reflected in the irregular shape of pulmonary vessels, especialy veins, when viewed in cross-section (Wagenvoort & Wagenvoort, 1977).

With regard to functional changes in the pulmonary circulation it seems that the effects of age and smoking may be insignificant. At rest Emirgil et al. (1967) could find no difference in the pulmonary vascular resistance of young (mean age 39) and aged (mean age 66) males, only during exercise was the pulmonary vascular resistance increased on average in the older age group, and even then there was considerable overlap between the two groups. Dexter et al. (1964) also could find no evidence of an increased pulmonary vascular resistance with increasing age in resting subjects. It would appear, therfore, that the effects of age and smoking on vessels in the pulmonary circulation are not of clinical significance.

Nevertheless, it is necessary to quantitate the effects of age and smoking so that changes in disease states may be viewed in perspective. For the purposes of this Introduction it is proposed that the two main structural components of pulmonary vessels, the media and intima be treated separately.

3.1.4 Alterations in the Medial Component of Pulmonary Vessels

Those researching the effects of age and smoking on the pulmonary vascular bed are mainly looking for an answer to this question - "Does medial hypertrophy (or hyperplasia) occur?" Predictably, the group of vessels that have received most attention are the muscular pulmonary arteries, although not to the exclusion of all other vessel types.

Starting wih a general comment it can be said that the changes in the pulmonary arteries are considerably more marked than those in the pulmonary veins. Qualitative changes have been reported in the media of pulmonary arteries with increasing age (specifically muscular pulmonary arteries) by Wagenvoort & Wagenvoort (1965a); these changes are such that the medial layer gradually becomes more irregular and fibrotic. This feature of ageing is one with which most workers would agree. There is much disagreement, however, about the occurrence of quantitative changes in the media of pulmonary arteries despite the fact that most workers have used methods of assessment similar in principle, namely expression of medial thickness in relation to vessel diameter. The majority of those studying uninjected arteries have concluded that there is no age effect, either on the large pulmonary arteries at the hilum of the lung (Mackay et al., 1978) or on the muscular pulmonary arteries (Wagenvoort & Wagenvoort, 1965a). However, when pulmonary arteries are distended with an injection medium and percentage medial thickness measurements made, arteries from aged lungs do appear to

have a thicker medial layer than arteries from young lungs (Semmens, 1970; Simons & Reid, 1969; Warnock & Kunzmann, 1977a).

Far less attention has been paid to the effects of smoking on pulmonary blood vessels and indeed in many of the studies mentioned in the preceding paragraph the smoking history of the subjects was not taken into account in the analysis of the data or interpretation of the findings. The reverse was the case in the study by Hale et al. (1980) in which the effects of age were ignored; the conclusions drawn from this particular study were that smokers have an increased number of arteries measuring less than 200μm in diameter, possibly due to an extension of muscle into previously non-muscularised vessels. Medial hypertrophy was also reported to be present in what were obviously existing muscular pulmonary arteries.

Although there seems to be uncertainty regarding the effects of ageing especially on the media of pulmonary arteries there are a number of perhaps incidental but not unimportant points on which there seems to be universal agreement. Simply stated there are no lobar, lung or sex differences with regard to the percentage medial thickness measurement (Simons & Reid, 1969; Wagenvoort & Wagenvoort, 1965a).

3.1.5 Alterations in the Intimal Component of Pulmonary Vessels

One of the most striking histological features of the adult pulmonary vasculature is that of intimal fibrosis. There is some debate as to whether this should be considered 'normal' or

'pathological'. On the one hand it is extremely common and is not linked to disease as such, but on the other hand its occurrence is limited to subjects living in the Western world (Wagenvoort & Wagenvoort, 1979). On balance most workers describe it as 'normal'.

Atheromatous patches of minimal or mild degree are found in the pulmonary trunk and main pulmonary arteries of most people aged forty or over (Brenner, 1935c) and are most frequently seen at branching points (Wagenvoort & Wagenvoort, 1979). The severity of atherosclerosis in these vessel types is rarely worse than mild unless pulmonary hypertension or hypercholesterolemia happens to be present. Patches of atheroma continue to be found with increasing age down to the level of the larger elastic pulmonary arteries, that is the lobar and segmental arteries; smaller elastic arteries tend not to be affected unless other disease is present. Again the branching points are the most commonly affected sites (Wagenvoort & Wagenvoort, 1979).

In the muscular pulmonary arteries the most common age change is that of intimal fibrosis, the prevalence of which increases dramatically with age although there is considerable individual variation in its presence and extent (Brenner, 1935b; Harris & Heath, 1977; Wagenvoort & Wagenvoort, 1965a). Generally speaking there is only a slight increase in the thickness of the intimal component up to the age of forty or so; thereafter intimal thickness shows a steep rise (Wagenvoort & Wagenvoort, 1965a) with an average of 50% of arteries becoming affected (Warnock & Kunzmann, 1977a). Most researchers have specifically stated that these intimal age changes in the muscular pulmonary arteries are most

common and most severe in the medium or smaller-sized vessels (Semmens, 1970; Wagenvoort & Wagenvoort, 1965a) and also that they have a predilection for the upper lobe (Hale et al., 1980; Wagenvoort & Wagenvoort, 1965a). Delarue et al. (1958) have suggested that the latter factor might be related to the low blood flow in the apex of the lung, which in theory could result in an increased tendency to thrombosis in periods of lung infections etc.

In addition to ageing, smoking has been implicated as a cause of increased intimal thickening in muscular pulmonary arteries (Auerbach et al., 1963; Hale et al., 1980). Unfortunately few studies of the effects of smoking have been carried out, as with the medial component the main thrust of the work has been to elucidate the effects of ageing.

The occurrence of intimal fibrosis with increasing age does not stop at the level of the muscular pulmonary arteries; thick layers of fibrosis are often seen in the walls of pulmonary arterioles in older individuals (Wagenvoort & Wagenvoort, 1977) and even the pulmonary veins are not exempt from this 'pathological' process (Wagenvoort & Wagenvoort, 1979).

This form of senile intimal fibrosis affecting vessels in the pulmonary circulation consists of a very narrow layer of collagen, frequently hyaline in appearance, which is normally acellular (Harris & Heath, 1977). Cellular proliferation, although rare, is sometimes seen in the muscular pulmonary arteries (Smith & Heath, 1980; Wagenvoort & Wagenvoort, 1965a), as is elastosis (Wagenvoort & Wagenvoort, 1965a). The cell involved in intimal fibrosis is a

myofibroblast which shares the properties of fibroblasts and smooth muscle cells (Smith & Heath, 1980).

One last but very important point with regard to intimal fibrosis concerns its distribution round the vessel wall. This point has been left to last quite intentionally because it ties in with comments made in the following section. Not only is intimal fibrosis patchy in terms of the number of vessels involved, cross-sectionally cut vessels also show that it rarely affects the whole wall of a vessel to a uniform extent. It is normally excentric in distribution (Wagenvoort & Wagenvoort, 1965a; Wagenvoort & Wagenvoort, 1977); often the patches of intimal fibrosis are described as being 'cushion-like' or 'crescent-shaped'. Unfortunately this distribution of intimal fibrosis may have a significant effect on the intimal thickness measurement which most workers have chosen to use in their assessment of intimal abnormality. The next section deals with methods used to assess the effects of age and smoking on the two major structural components of pulmonary vessels, and what the advantages and disadvantages of these methods are; in the latter section the problems brought about by the generally irregular distribution of intimal abnormality are expanded upon.

3.1.6 Methods Used to Assess the Effects of Age and Smoking on Pulmonary Vessels

It is true to say that few studies have used methods other than the simple percentage medial or intimal thickness methods. Exceptions are studies such as those of Naeye & Dellinger (1971) in

which the relative proportions of particular tissue components in the vascular wall are measured. While this approach has its merits it does not assess whether medial hypertrophy has occurred or not, and neither does it assess the degree of intimal abnormality, which are really the two most important factors that need to be established.

While simple subjective grading methods have been used to assess changes in the media and intima of arteries, e.g. Auerbach et al. (1963), the vast majority of workers have quantitated the changes by the 'wall thickness' methods mentioned at the beginning of this section. Differences between workers relate mainly to whether the pulmonary arteries of the specimens have been distended by an injection medium or not. The advantages of these methods are that the measurements are easily obtained and, if there are no objections to assuming that the shorter diameter is representative of the size of vessels cut obliquely, then all vessels can be measured. However, the disadvantages of these methods far outweigh their advantages. These disadvantages have already been covered in detail in Chapter 2 so it is sufficient to simply re-emphasise the main problems here. In uninjected arteries there is the problem of the variation in the amount of artery collapse or constriction which affects the thickness of both the media and the intima, and also the diameter of the vessel. In addition, the distribution of intimal abnormality round the vessel wall may be such that the intimal thickness at the measured points is unrepresentative of the vessel as a whole. Injection of vessels is not an ideal solution to these problems owing to the difficulties involved in deciding what

pressures should be used and the influence of intimal abnormality on the distensibility characteristics of vessels (Warnock & Kunzmann, 1977a).

To the author's knowledge there have been no studies of the effects of age and smoking on pulmonary vessels, which have used methods that are independent of vessel constriction/collapse and the generally irregular distribution of intimal abnormality. Because of this it has to be said that the effects of age and smoking have not been satisfactorily established, and that there is still a great deal of useful work to be done in this area.

3.2 AIMS

The main aims of this chapter were as follows:-

1. Using a suitable study group to measure all cross-sectionally cut muscular pulmonary arteries in each subject using the techniques developed in Chapter 2.

2. For each subject to investigate the relationship between measures of both the medial and intimal components of arteries and their size, the purpose of this being to determine what were the most sensible summary data to use for the arteries in each subject as a whole.

3. Using the summary data for the media and intima to investigate, as a starting point, the effects of age and smoking on these two vascular components.

3.3 MATERIAL AND METHODS

3.3.1 The Subjects

It was originally intended that the study of the effects of age and smoking on muscular pulmonary arteries be carried out using lung tissue resected from patients with small peripheral carcinomas. For a variety of reasons, discussed later, the study was extended to include material obtained from routine autopsies. A description of the subjects included in these two groups is given below.

(i) Resection group

From Sepember 1980 to May 1984 material was obtained from resection operations carried out at the City Hospital, Edinburgh on patients with suspected lung carcinomas. This material was supplied to Alec McLean of the Department of Pathology, University of Edinburgh for use in his morphometric studies of small airways; permission to use the material in the present study was given by David Flenley, Professor of Respiratory Medicine, and Dr David Lamb, also from the Department of Pathology.

Thirty-two specimens were offered to the author for study of the pulmonary arteries. The sex, age and smoking hisory of these 32 patients is given in Table 3.1 which shows that the majority (25) of the patients were male and that current smokers predominated (28); only one life-long non-smoker was included. The patients' ages ranged from 46 to 72 years. Table 3.1 also shows that four whole lungs were obtained and that the lobectomy specimens comprised 18 upper lobes, three middle lobes and seven lower lobes.

Table 3.1 A description of the 32 subjects in the resection group.

Subject Number	Sex	Age	Smoking Status	Material* Available
1	M	47	NS	RUL
2	F	52	S	RL
3	M	54	S	RL
4	M	56	S	RML
5	M	61	S	RUL
6	M	63	S	RUL
7	M	51	S	RL
8	M	66	S	RLL
9	M	47	S	LLL
10	M	63	S	RUL
11	M	63	S	RUL
12	F	51	S	LLL
13	M	72	S	LUL
14	M	65	EXS	RLL
15	M	59	S	RLL
16	M	74	EXS	RML
17	M	62	S	LUL
18	M	68	S	RLL
19	M	63	EXS	LLL
20	F	57	S	LUL
21	M	52	S	LUL
22	M	66	S	LUL
23	M	57	S	LUL
24	M	65	S	RL
25	M	58	S	LUL
26	F	56	S	RUL
27	M	70	EXS	RUL
28	F	59	S	RUL
29	F	66	S	RUL
30	F	54	S	LUL
31	F	46	S	RML
32	M	64	S	LUL

* LUL = Left upper lobe

LLL = Left lower lobe

RUL = Right upper lobe

RML = Right middle lobe

RLL = Right lower lobe

RL = Right lung

Additional data were available for the 32 patients, some of which were considered relevant to the present study namely: height, amount smoked (current and ex-smokers), years smoked (current and ex-smokers) and years ex-smoker (where relevant). In all cases the smoking histories were obtained directly from the patient. Ex-smokers were defined as those who had given up smoking at least four months prior to their resection operation.

Finally, pathology reports were available detailing the findings of macroscopic and microscopic examination of each resection specimen.

(ii) Autopsy group

Owing to the difficulties involved in trying to collect a suitable study group in the time available extensive use was made of post-mortem material that had been prepared prior to the start of the present study in 1981; it was provided by Dr David Lamb of the Department of Pathology, University of Edinburgh. The material, which consisted of tissue blocks taken from the mid-sagittal slice of left lungs, had been collected by Dr Lamb for a study of the effect of smoking on small airways making it suitable for the present study, the subjects concerned covering a wide age range with all smoking habit groups represented.

Twenty-three subjects were included in the present study, none of whom had any disease in either the lungs or heart which might have affected the pulmonary vasculature. Two age groups were specified, less than 35 and 50 to 70 years, and all subjects falling

into either of those two groups, who had a complete smoking history, were selected for study. To extend the study of the effects of ageing alone on muscular pulmonary arteries, a further four non-smokers aged over 70 were also included.

Table 3.2 gives details of the sex, age and smoking history of the 23 subjects. As with the resection group the majority of the subjects were male (21). Unlike the resection group, however, life-long non-smokers were well represented, comprising just over half of the study group; there were no ex-smokers. The age range of the subjects was 19 to 81 years.

A vast array of additional clinical and pathological data were available for each subject. Those data thought relevant to the present study were: height, amount smoked, years smoked and occupation. For all subjects the smoking histories were obtained from the next of kin. With regard to the pathological data available, which included the cause of death and pathological findings in the lungs and heart, the following were considered important: the percentage of the lung (specifically the mid-sagittal slice) involved in both panacinar and centriacinar emphysema, the number of centriacinar lesions present, the weights of both the left ventricle plus septum and the right ventricle, and the Gland:Wall ratio of the main bronchus (a mean of three values).

3.3.2 Preparation of Specimens

As all tissue preparation methods have been extensively described in Chapter 2 it is proposed that only a brief summary be

Table 3.2 A description of the 23 subjects in the autopsy group.

Subject Number	Sex	Age	Smoking Status
1	M	29	S
2	M	25	NS
3	M	19	S
4	F	25	S
5	M	31	NS
6	M	62	NS
7	M	51	S
8	M	61	NS
9	M	66	S
10	M	64	NS
11	M	52	NS
12	M	62	S
13	M	55	S
14	M	61	S
15	M	56	S
16	M	59	S
17	M	58	S
18	M	65	NS
19	M	53	NS
20	F	81	NS
21	M	70	NS
22	M	78	NS
23	M	73	NS

given here; details may be obtained from the sections referred to in brackets.

(i) Procedure for resection specimens

Following inflation through the main or lobar bronchus by the routine method (section 2.3.3) lungs or lobes were sliced at 1cm intervals in the sagittal plane and 12 standard tissue blocks taken from the two most lateral slices, using a stratified random sampling technique (section 2.3.4). Following embedding in glycol methacrylate (section 2.3.5 (ii)), the blocks were sectiond at 3μm (section 2.3.6) and stained for elastic (section 2.3.7).

(ii) Procedure for autopsy specimens

The left lungs were routinely inflated through the main bronchus (section 2.3.3) and sliced at 1cm intervals in the sagittal plane. In general 12 tissue blocks were randomly selected from the mid-sagittal slice, six from each of the upper and lower lobes (section 2.3.4). These blocks were embedded in paraffin wax (section 2.3.5 (i)), sectioned at 5μm (section 2.3.6) and stained using either Weigart's or Miller's elastic stain with a van Gieson counterstain (section 2.3.7).

3.3.3 Method of Measurement

The histological sections of all subjects were scanned and each cross-sectionally cut muscular pulmonary artery identified. Those with a well-defined internal elastic lamina (the 'digitisable' arteries) were measured twice in succession, once using the standard technique associated wih Program 1 and once using the abridged

technique. These two procedures are described in detail in sections 2.3.10 and 2.3.11 respectively . Cross-sectionally cut arteries with an ill-defined internal elastic lamina were measured only once, using the abridged technique. After measuring each artery its crinkle grade was recorded (obtained as described in section 2.3.12).

On completion of the artery measurements for each subject calculations were done using data from the 'digitisable' arteries to determine how much longer on average was the total length of the internal elastic lamina compared to the length of the boundary between intima and media for arteries assigned a crinkle grade of 0, 1, 2, 3 and 4. These factors were then used to estimate the total length of the internal elastic lamina in the 'undigitisable' cross-sectionally cut arteries.

3.3.4 Analysis of Data

The procedures adopted in this chapter were different from those used in the previous chapter. Only limited use was made of the statistical program 'Simple Regressions' (described in section 2.3.14) for analysing the relationship between any two variables. Overall, the micro-computer associated with the digitising system was little used. Instead the individual artery data, and the clinical and pathological data for each subject were transferred to tape on the main-frame computer (a PRIME 750) at the Institute of Occupational Medicine. To start with, exploratory analyses were carried out on the relationship between measures of both the medial and intimal components of arteries and their size for all subjects.

The purpose of these analyses was to determine what were the most sensible summary data for the media and intima to use in the investigation of the effects of age and smoking. All these analyses were carried out using the statistical package 'Minitab' which is a very flexible and powerful statistical computing system. Details of this system may be found in the Minitab Reference Manual (Ryan et al., 1982a) and the Minitab Student Handbook (Ryan et al., 1982b)). Its only drawback is that it can only carry out linear regressions so in all analyses the linearity of the regression lines had to be checked; in cases where the fit was not linear then the data were reanalysed using the statistical program 'Simple Regressions' (described in section 2.3.14) in conjunction with the digitiser set-up.

3.4 RESULTS

3.4.1 Preliminary Analysis of Artery Data for Individual Subjects

In order to investigate the effects of age and smoking on muscular pulmonary arteries it was first of all necessary to produce summary data for the media and intima, which would be representative of the arteries in each subject as a whole. To determine which summary data ought to be used was the main purpose of the preliminary analysis. As a first step it was proposed that the relationship between both medial area (square root of) and Intima Index and artery size be investigated for each subject in the resection and autopsy groups. In addition to deciding on the most sensible summary data to use, it was also proposed to determine whether different 'classes' of artery needed to be treated separately; particular attention was paid to possible differences between 'digitisable' and 'undigitisable' cross-sectionally cut arteries, and also to possible differences between arteries from different lobes.

(i) The media

Using Minitab the square root (necessary to linearise the relationship - see section 2.4.6) of medial area was plotted against the total length of the internal elastic lamina for all measured cross-sectionally cut muscular pulmonary arteries in each subject; the linearity of the regression line was checked in each case. One of the most striking points to emerge from these plots was the enormous variation in the size of the largest measured artery,

values for length of internal elastic lamina ranging from 1475μm to 4179μm. This variation is explained by the fact that in some subjects none of the larger arteries were cross-sectionally cut. On account of this it was concluded that the effects of age and smoking could only be fully investigated in arteries measuring up to 1475μm (for convenience this was increased to 1500μm). However, the vast majority of subjects with a limited size range of measured arteries were in the autopsy group, a factor probably linked to the paraffin embedding of tissue in this group. In the resection group it was considered that the size range could be extended to 3000μm length of internal elastic lamina. Using these two cut-off points the plots of square root of medial area against artery size were repeated, and again the linearity of the regression lines was checked. For the autopsy group two plots were done per subject, one investigating possible differences between 'digitisable' and 'undigitisable' cross-sectionally cut arteries, the other possible differences between arteries from different lobes. From these investigations it appeared that there was no need to account for 'different' classes of artery separately. This is illustrated for one subject in the autopsy group, subject 16, in Figures 3.1 and 3.2. It can be seen from these two figures that the 'digitisable' and 'undigitisable' arteries behave similarly, as do the upper and lower lobe arteries.

With regard to the resection group there also appeared to be no obvious differences between 'digitisable' and 'undigitisable' arteries in either the arteries up to 1500μm or 3000μm length of internal elastic lamina. Figures 3.3 and 3.4 illustrate this point for subject 2 in the resection group. It was not possible to

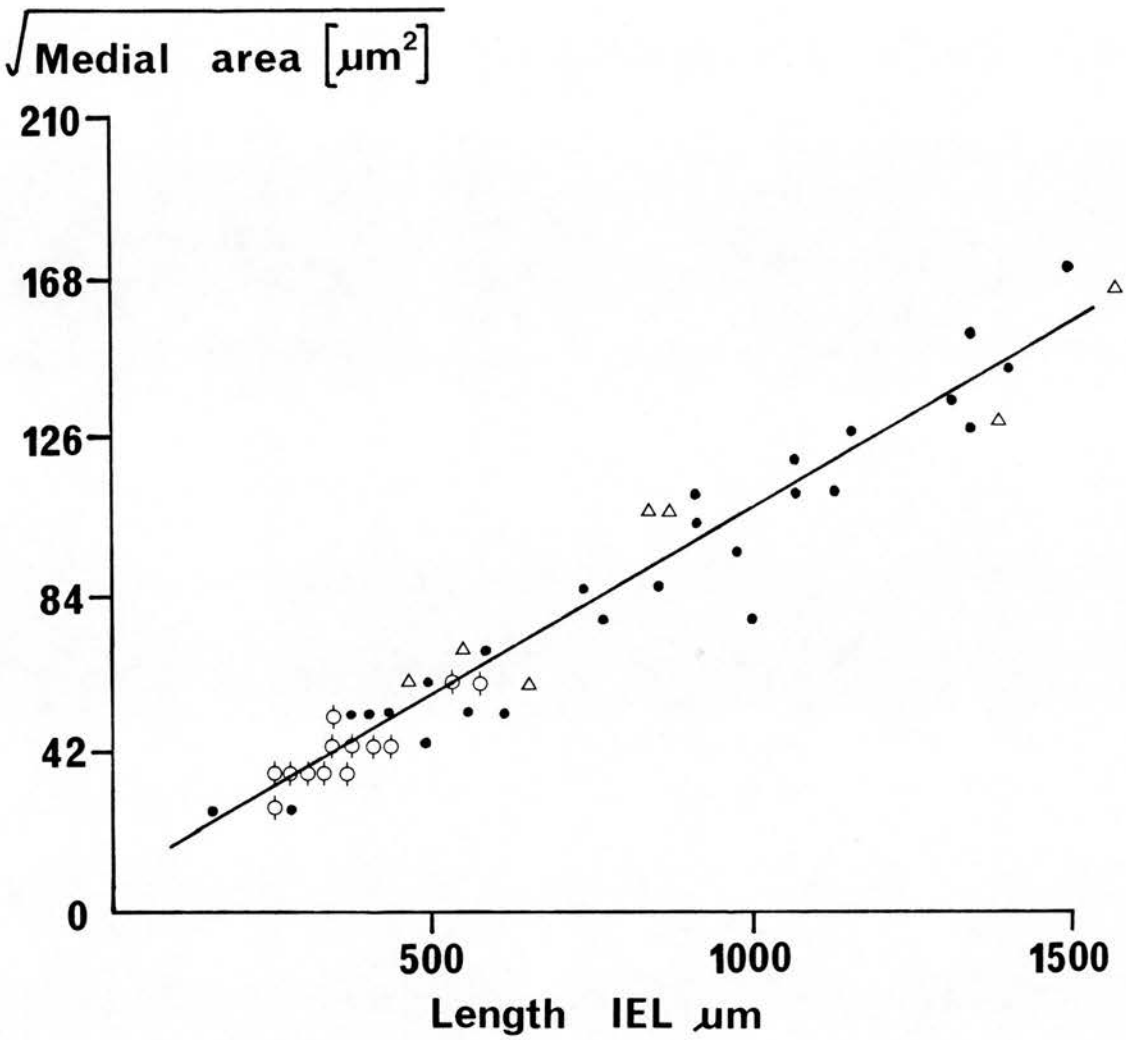


Figure 3.1 The relationship between medial area (square root of) and artery size in subject 16 of the autopsy group. 'Digitisable' (•) and 'undigitisable' (△) cross-sectionally cut arteries separately identified. ϕ = coincident points.

Line of best fit: $y = 7.81 + 0.101x$, $r = 0.98$

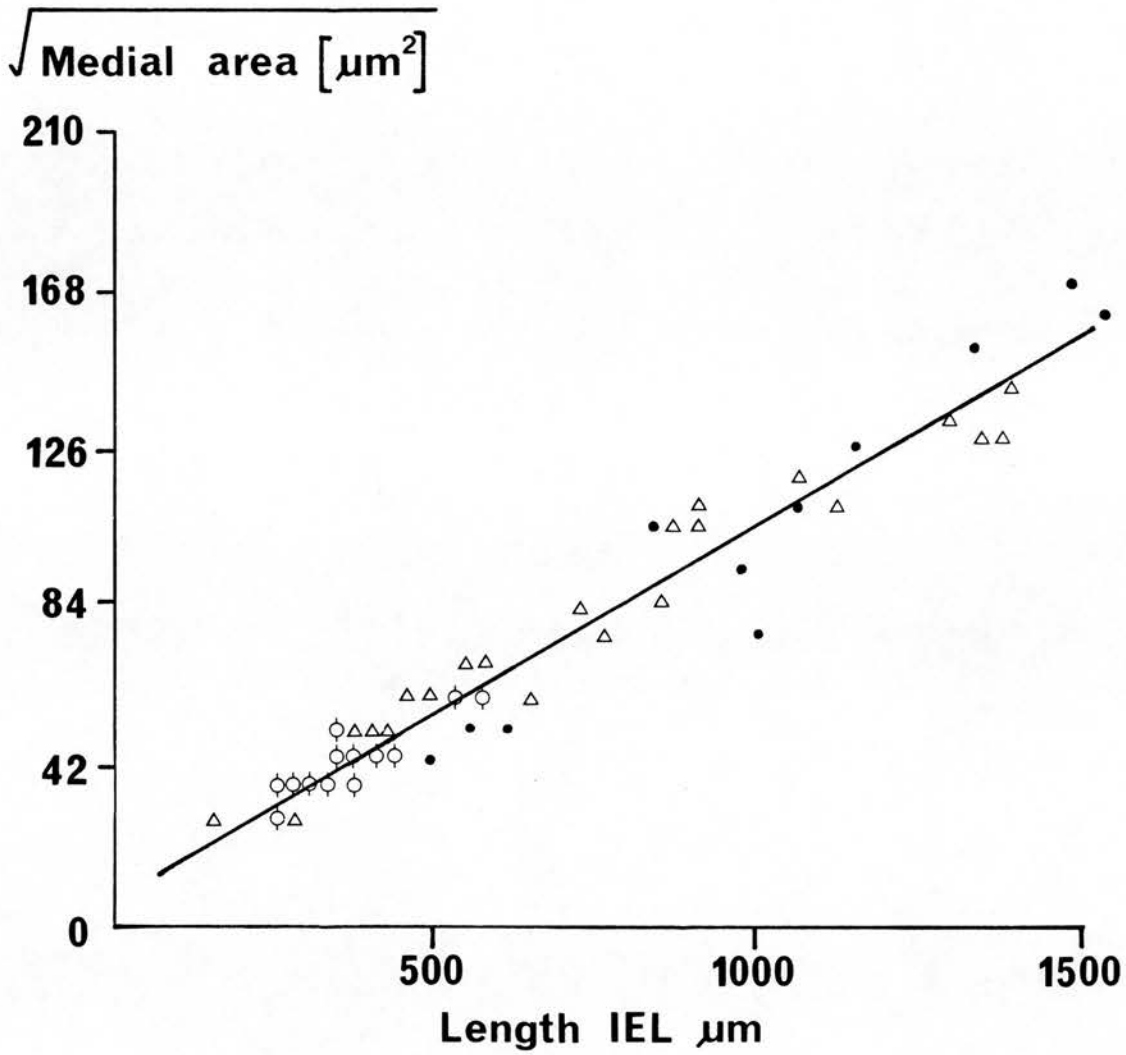


Figure 3.2 The relationship between medial area (square root of) and artery size in subject 16 of the autopsy group. Upper (•) and lower (△) lobe arteries separately identified. ϕ = coincident points.

Line of best fit: $y = 7.81 + 0.101x$, $r = 0.98$

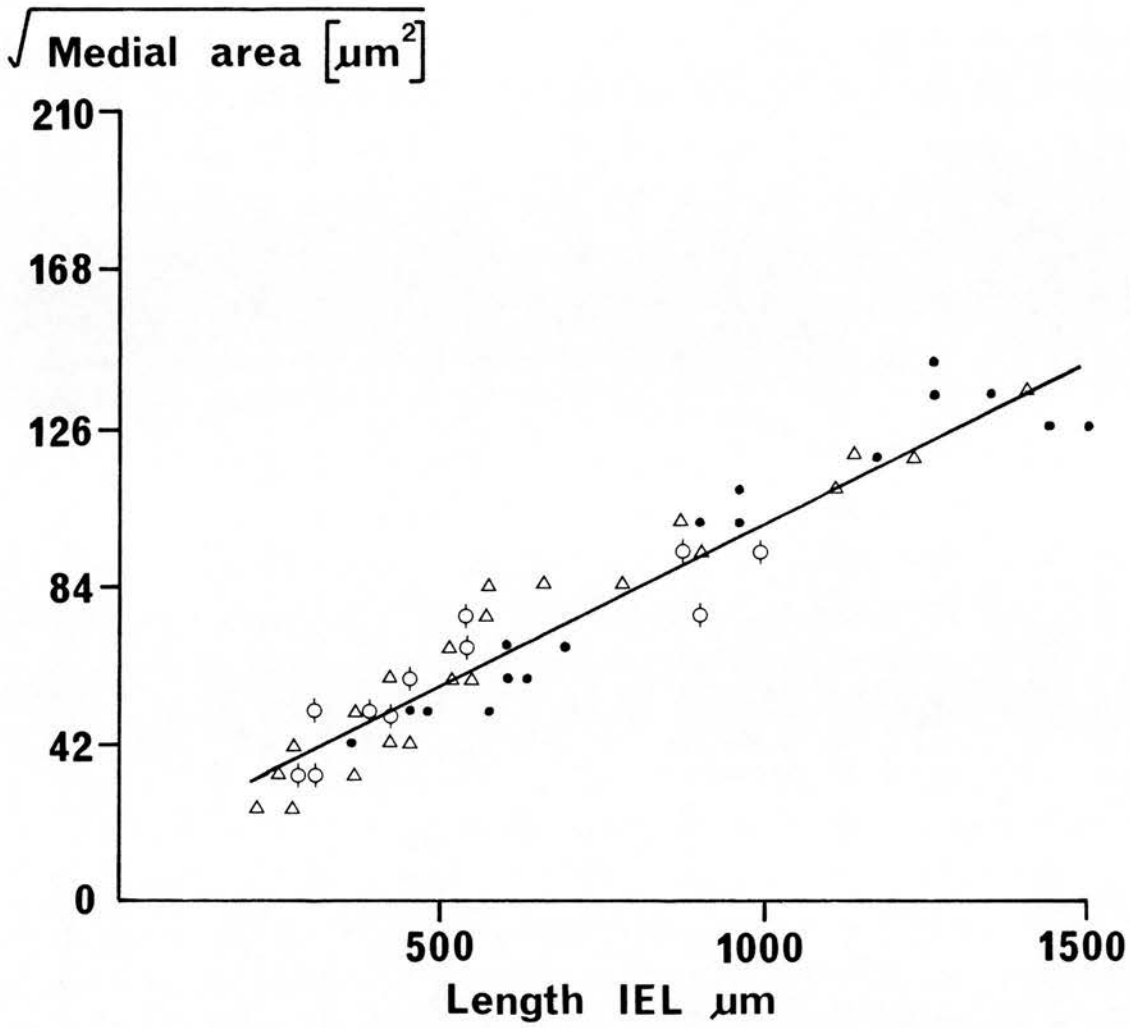


Figure 3.3 The relationship between medial area (square root of) and artery size (up to 1500μm) in subject 2 of the resection group. 'Digitisable' (•) and 'undigitisable' (△) arteries separately identified. ϕ = coincident points.

Line of best fit: $y = 14.8 + 0.087x$, $r = 0.97$

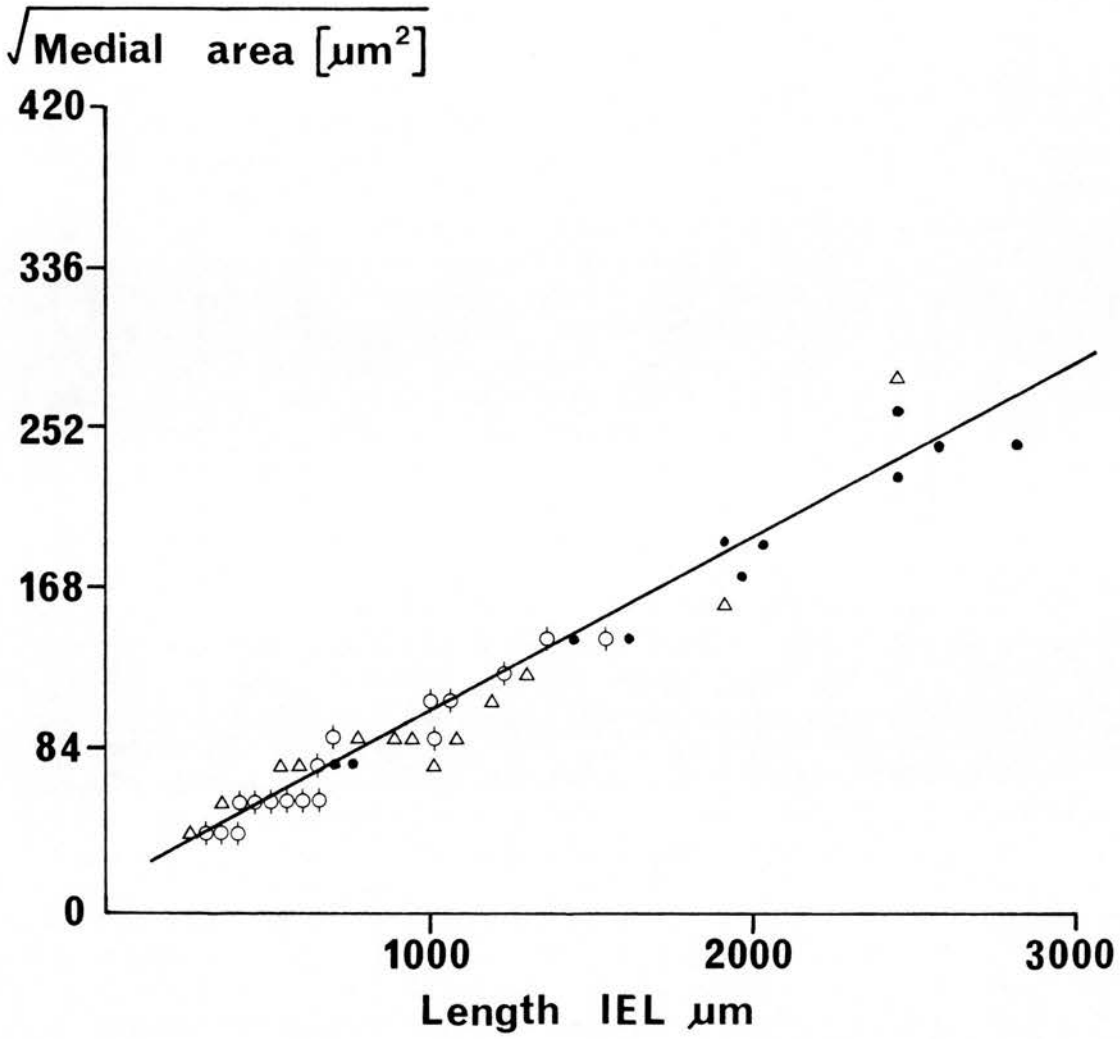


Figure 3.4 The relationship between medial area (square root of) and artery size (up to 3000μm) in subject 2 of the resection group. 'Digitisable' (•) and 'undigitisable' (△) arteries separately identified. ϕ = coincident points.

Line of best fit: $y = 10.7 + 0.094x$, $r = 0.98$

investigate lobar differences in medial area measurements in the resection group since nearly all of the specimens comprised single lobes.

For all subjects in both the autopsy and resection groups the slopes of the regressions of square root of medial area against artery size were highly significantly ($p<0.001$) different from zero. Also the fit of individual data points to these lines was extremely good as indicated by the values for the correlation coefficients. For these reasons it was thought that the slopes of the regression lines (slope 1500, slope 3000 values) might be a useful way of summarising data on the medial component of pulmonary arteries for each subject, thus allowing the effects of age and smoking on this vascular component to be studied. On further reflection, however, it was concluded that the use of slopes might be a somewhat difficult concept to grasp so the following approach was also included as an alternative method of summarising the data on the media, a method which, it was hoped, would yield more readily comprehensible results. Using the linear regression equations, it was proposed to 'predict' for each subject the medial area (square root of) values for arteries of a specific size. The size points chosen were 500, 1000, 1500, and, in the case of the resection group, 3000μm length of internal elastic lamina; these medial area (square root of) values were termed MA500, MA1000, MA1500 and MA3000. The idea behind this alternative method to using the slopes as a means of summarising data on the media was that the effects of age and smoking could be looked at in arteries of specific sizes thereby yielding additional useful information on the effects of

these two factors on the medial component of muscular pulmonary arteries.

(ii) The intima

In Chapter 2 study of a group of subjects with varying degrees of intimal abnormality had shown that this was best expressed in the form of an Intima Index (section 2.4.14). Furthermore, due to differences between subjects in terms of the relationship between Intima Index and artery size it was concluded that subjects were best compared by calculating mean Intima Indices for arteries subdivided into size groups (section 2.4.14). The purpose of the preliminary analysis in the present section was to determine what these size groups should be. An additional aim was to establish whether it was necessary to treat different 'classes' of artery separately, the classes in question being 'digitisable' and 'undigitisable' cross-sectionally cut arteries, and arteries from different lobes.

Intima Index was plotted against total length of internal elastic lamina for all measured arteries of each subject in the autopsy and resection groups using Minitab. For all subjects the relationship between these two variables was similar, the value for Intima Index tending to increase with decreasing size of artery, which confirms earlier results (section 2.4.14). This result is illustrated for subject 16 in the autopsy group and subject 2 in the resection group in Figures 3.5 and 3.6. One other point is illustrated in these two figures, which is that there are no consistent or obvious differences between 'digitisable' and

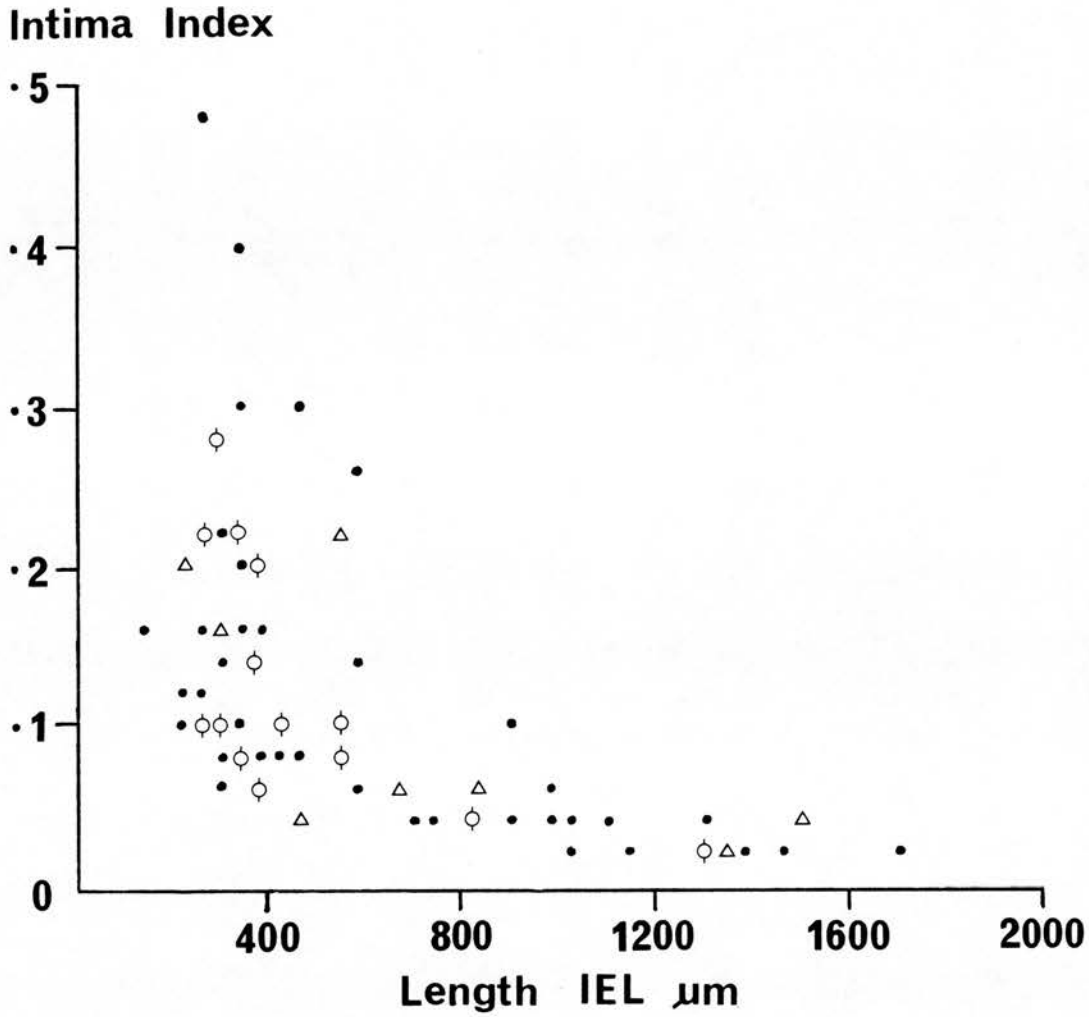


Figure 3.5 The relationship between Intima Index and artery size for subject 16 in the autopsy group. 'Digitisable' (•) and 'undigitisable' (△) cross-sectionally cut arteries separately identified. ϕ = coincident points.

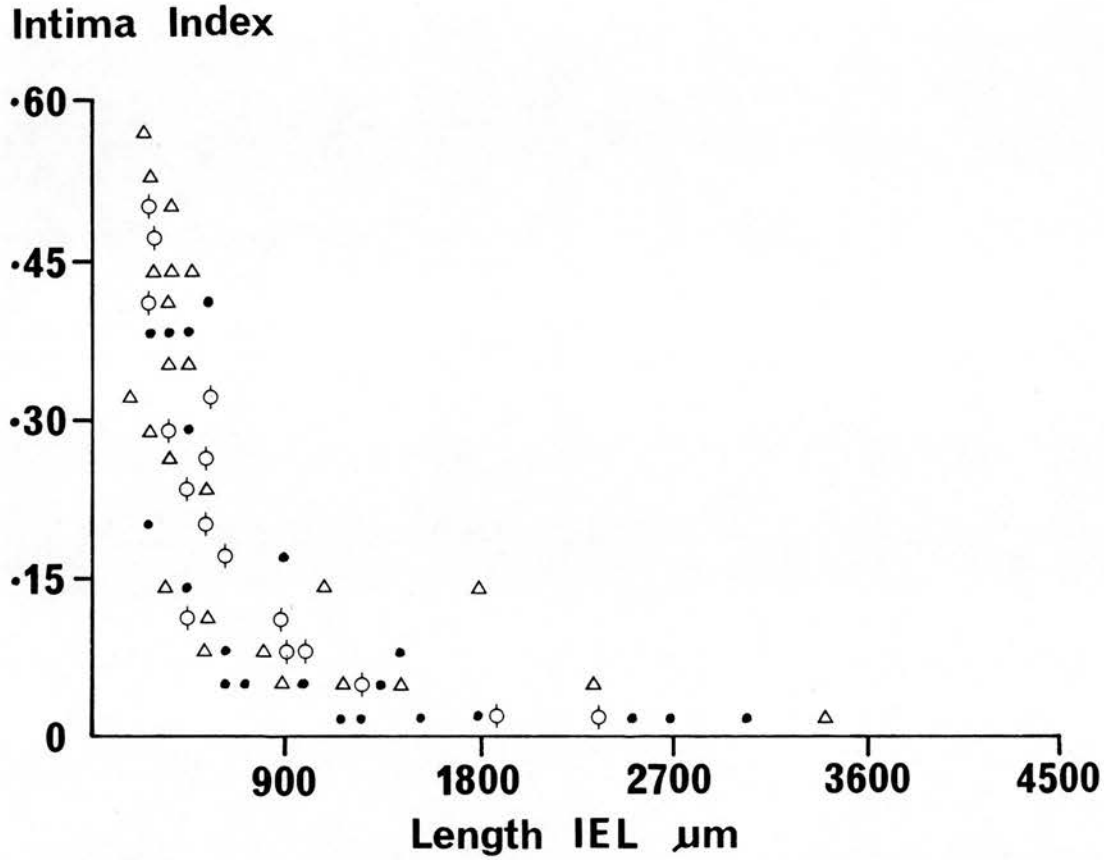


Figure 3.6 The relationship between Intima Index and artery size for subject 2 in the resection group. 'Digitisable' (•) and 'undigitisable' (△) cross-sectionally cut arteries separately identified. ϕ = coincident points.

'undigitisable' arteries making it unnecessary to account for them separately.

Regarding possible differences in the Intima Indices of arteries from different lobes, study of subjects in the autopsy group led to the conclusion that there were none. Taking subject 15 as an example it can be seen (Figure 3.7) that there is nothing to suggest that there are any consistent differences in the Intima Indices of upper and lower lobe arteries.

The decision on what size groups to use was made with a view to satisfying these two criteria: an acceptable number of arteries in each group, and cut-offs at points where the relationship between Intima Index and artery size appeared to be changing. On examining the data for individual subjects in the two study groups the following size groupings were thought to provide the best possible compromise: $\leqslant$600μm, 601-1200μm, 1201-1800μm and >1800μm total length of internal elastic lamina. For each subject mean Intima Indices of arteries in these size groups were calculated using Minitab. These mean values are referred to as II600, II1200, II1800 and II>1800 respectively, the figures denoting the upper limit of the size range of arteries included in each group.

(iii) <u>The summary data</u>

The outcome of the preliminary analyses carried out in this section may be summarised as follows: the effects of age and smoking on muscular pulmonary arteries were to be investigated by exploring the relationship between these two factors and the summary data for the media and intima. For the media this was defined as

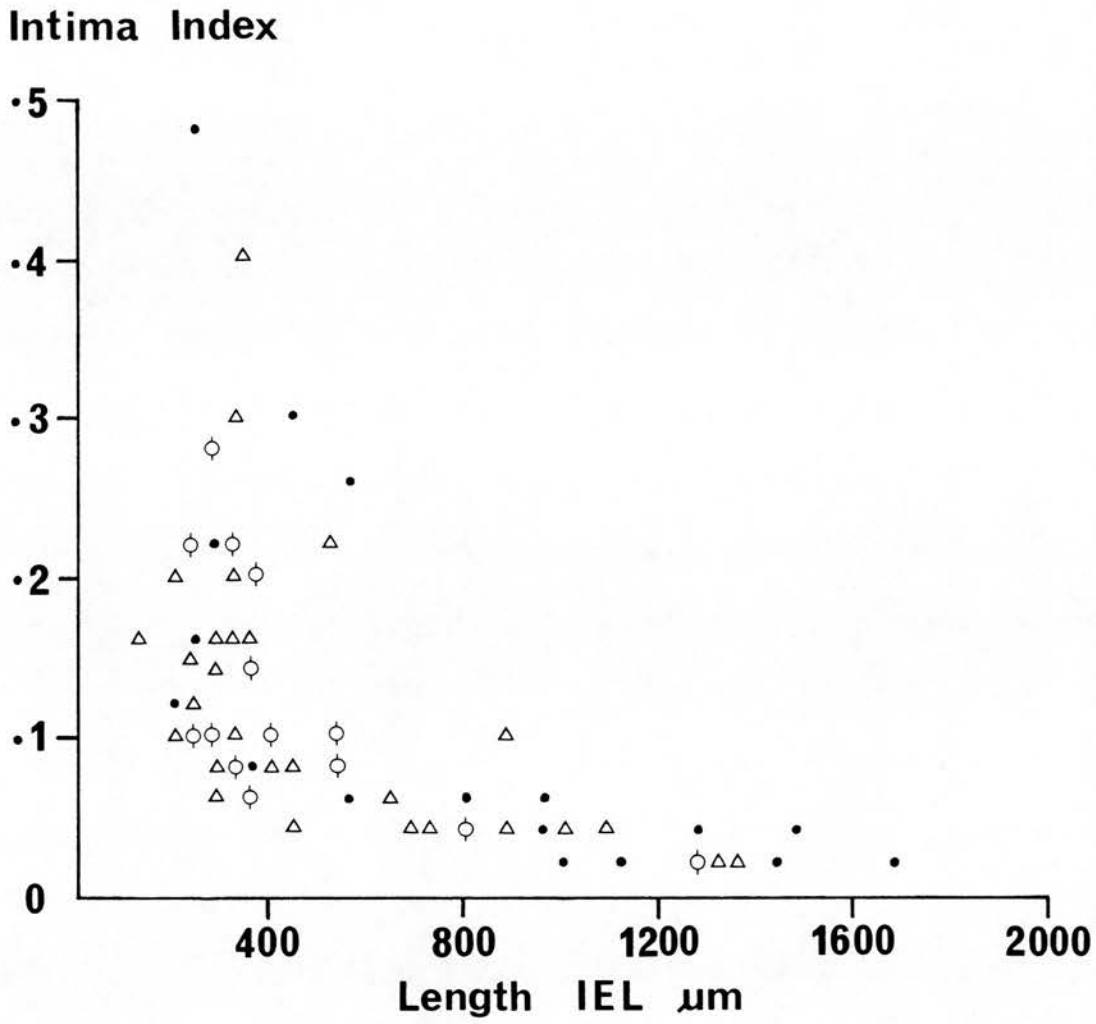


Figure 3.7 The relationship between Intima Index and artery size for subject 16 in the autopsy group. Upper (•) and lower (△) lobe arteries separately identified. ϕ = coincident points.

the slope of the regression line between square root of medial area and artery size, and additionally 'predicted' medial area (square root of) values for four specific sizes of artery. The summary data for the intima was defined as mean Intima Indices for arteries in four size groups.

3.4.2 The Effects of Age and Smoking on the Medial Component of Muscular Pulmonary Arteries

For all analyses the intention was to identify males and females separately in an effort to ascertain whether or not there were any sex differences. However, this was considered a pointless exercise in the autopsy group where only two of the 23 subjects were female.

(i) The autopsy group

Regression of the slope 1500 values against age revealed that there was no age effect on the medial component of muscular pulmonary arteries measuring up to 1500μm total length of internal elastic lamina, for the study group as a whole, or for non-smokers and smokers taken separately (Figure 3.8). At all ages the slopes of the regression lines between square root of medial area and artery size varied enormously, indicating in effect a considerable individual variation in the amount of muscle associated wih any given size of artery; overall, values for slopes ranged from 0.063 to 0.116.

Calculation of mean values for slope 1500 for non-smokers and smokers produced values of 0.091 and 0.088 respectively, which were

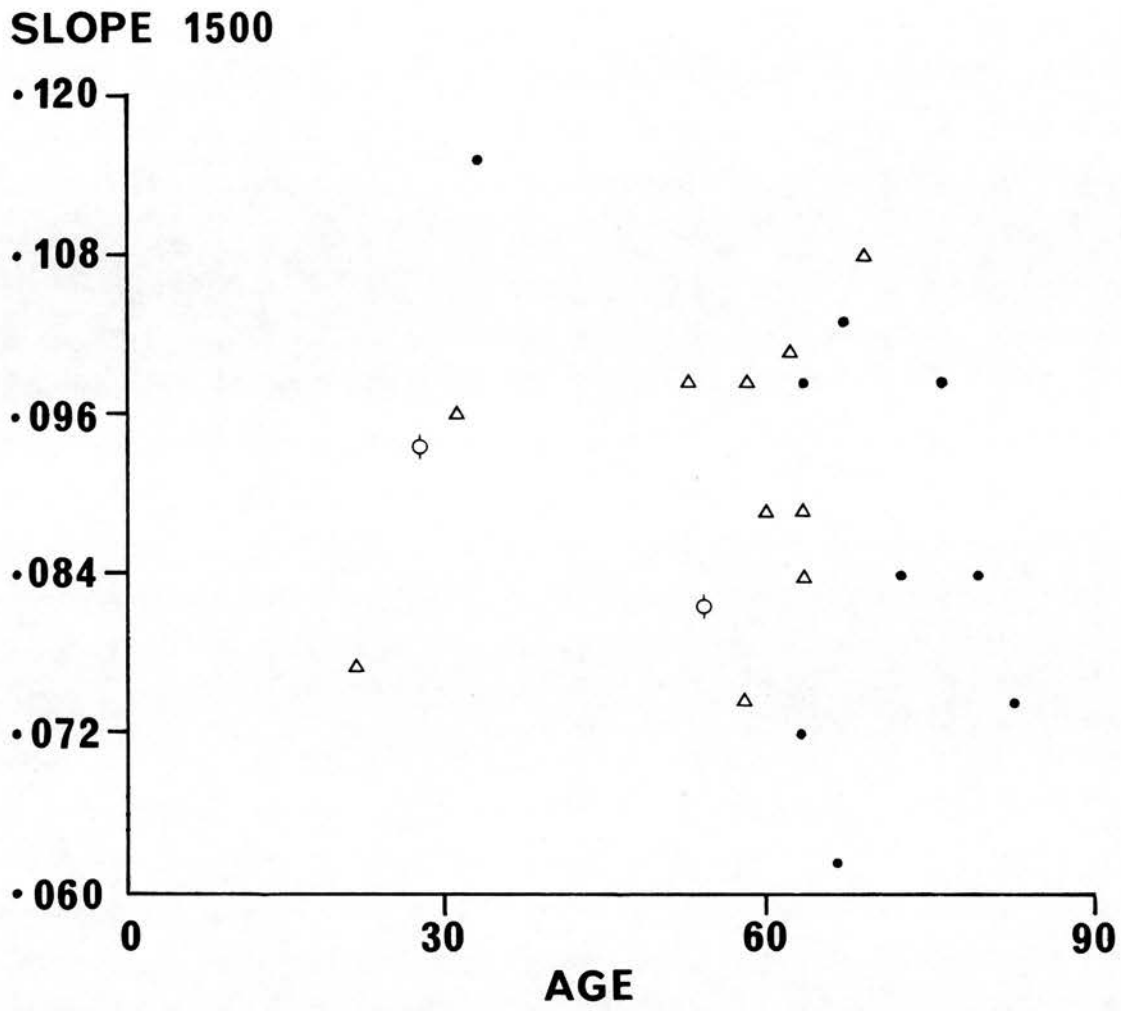


Figure 3.8 The relationship between the slope 1500 values for the media of muscular pulmonary arteries and age in subjects in the autopsy group. Smoking habit groups separately identified.

. = Non-Smokers

△ = Smokers

ϕ = Coincident points

not significantly different, indicating that there were no differences between non-smokers and smokers with respect to this parameter.

The lack of an age or smoking effect on the medial component of pulmonary arteries was confirmed by additional analyses using the alternative summary data for the media, namely the 'predicted' MA500, MA1000 and MA1500 values. The plots of MA500 against age are illustrated in Figure 3.9. At any age there was a marked individual variation in the amount of muscle in the wall of an artery measuring 500μm length of internal elastic lamina, which was not affected by smoking habit. The MA1000 and MA1500 plots against age showed a similar picture (result not illustrated).

(ii) The resection group

The foregoing analyses were repeated for the resection group this time separately identifying males and females in addition to the different smoking habit groups. Although the subjects in the resection group covered a much narrower age range than those in the autopsy group, Figure 3.10 shows that at any age there was an enormous variation in the slope 1500 values, individual values covering a range 0.067 to 0.114. These findings mirror those of the autopsy group.

This lack of an age effect on the medial component of pulmonary arteries with an internal elastic lamina measuring up to 1500μm was evident in both males and females (Figure 3.10) and also in ex-smokers and current smokers (result not illustrated). Furthermore there were no significant differences between males and females or

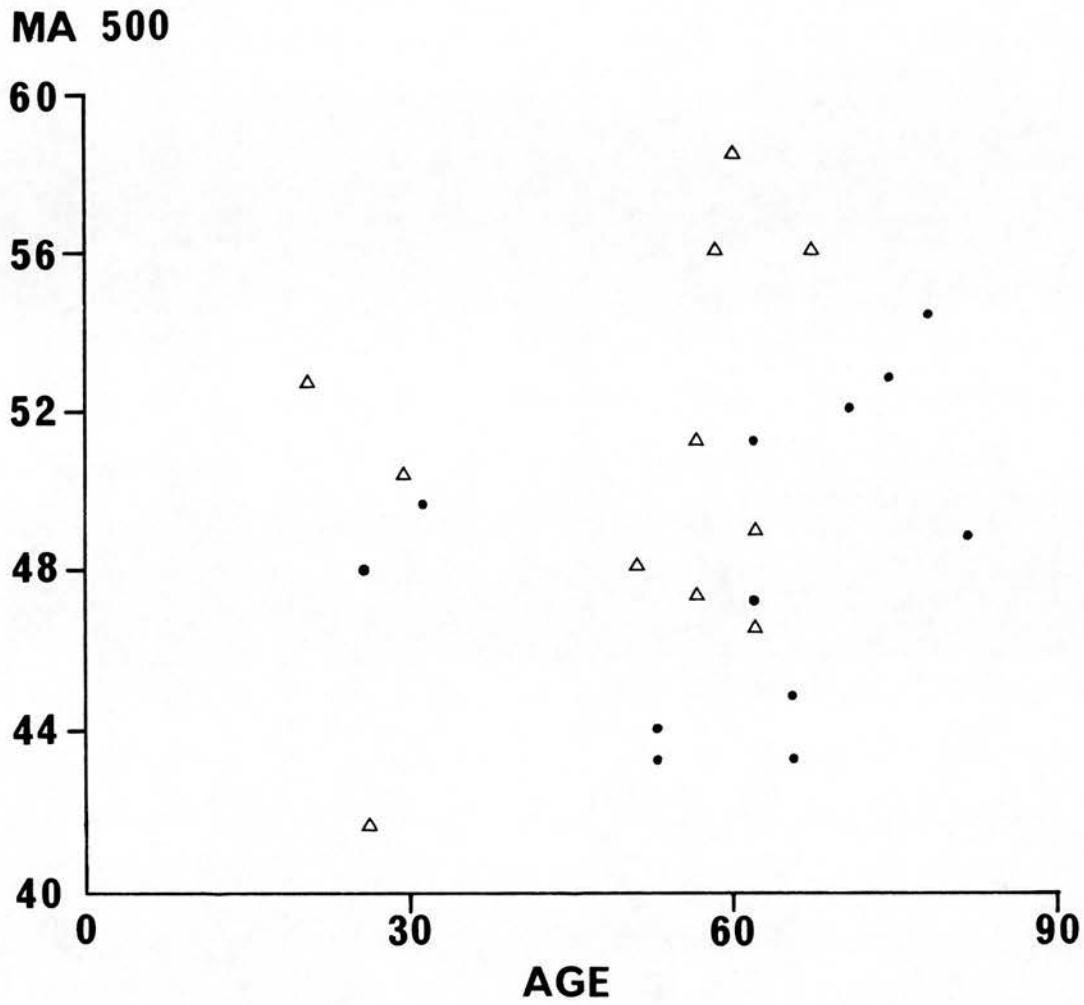


Figure 3.9 The relationship between estimated medial area values for arteries measuring 500μm (length of internal elastic lamina) and age in subjects in the autopsy group. Smoking habit groups separately identified.

. = Non-Smokers

△ = Smokers

ϕ = Coincident points

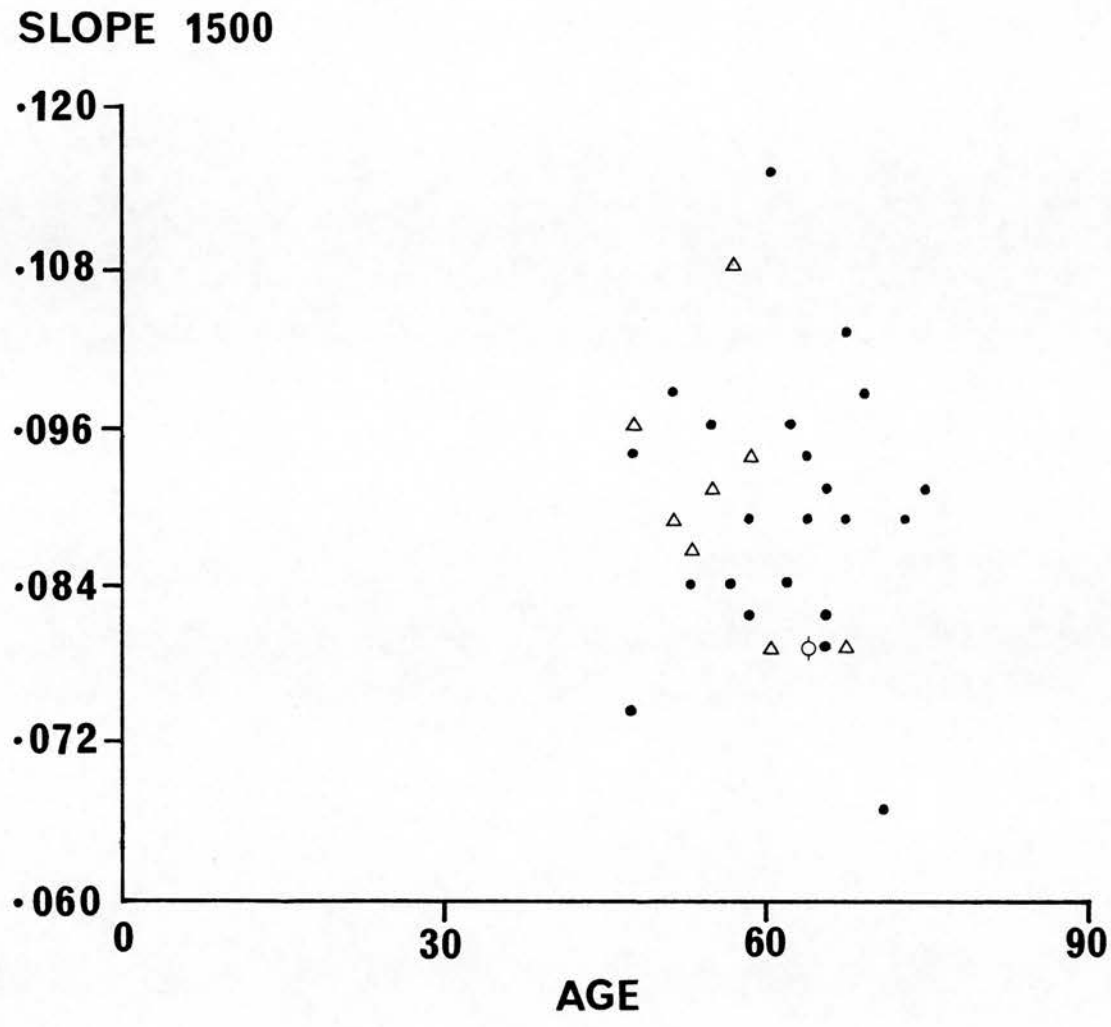


Figure 3.10 The relationship between the slope 1500 values for the media of muscular pulmonary arteries and age in subjects in the resection group. Males (·) and females (△) separately identified.

ϕ = Coincident points

between ex-smokers and smokers with respect to mean values of slope 1500.

When these analyses were repeated using the slopes of the regression line between square root of medial area and length of internal elastic lamina in arteries measuring up to 3000μm (slope 3000 values) there was still no evidence of an age effect on the medial component, and again there was nothing to suggest any differences between males and females or between ex-smokers and smokers (results not illustrated).

As with the autopsy group additional analyses using the alternative summary data for the media, the 'predicted' MA500, MA1000, MA1500 and MA3000 values, confirmed that for each of these artery size points there was a distinct individual variation in the amount of muscle in the artery wall. This was evident at all ages and was true regardless of the sex of the patient or his/her smoking habit (results not illustrated).

3.4.3 The Effects of Age and Smoking on the Intimal Component of Muscular Pulmonary Arteries

In addition to investigating the effects of age and smoking, males and females were separately identified, in the resection group only, to determine whether there were any sex differences.

(i) The autopsy group

For each subject mean Intima Indices were calculated for muscular pulmonary arteries in the four specified size groups;

these mean values are termed II600, II1200, II1800 and II>1800. Again using Minitab the mean Intima Indices were plotted against age for the whole group, and for non-smokers and smokers separately. The linearity of the regression lines was checked in all cases and in instances where the fit was not linear the data were re-analysed using the statistical program 'Simple Regressions' in conjunction with the micro-computer linked to the digitising system. The function giving the best fit was thus obtained.

The results of these analyses are shown in Figures 3.11 - 3.14, each figure representing one of the four size groupings. Beginning with a general comment about the group as a whole, and ignoring smoking habit differences for the time being, these figures (3.11 - 3.14) illustrate that for all four size groups of muscular pulmonary arteries the mean Intima Index increased with increasing age, although not always in a linear fashion, and not always to an extent that was statistically significant; only for the II600 (Figure 3.11) and II1200 (Figure 3.12) groups was there a significant increase with increasing age ($p<0.01$ and $p<0.05$ respectively).

Another general comment may be made concerning the range of the mean Intima Indices observed at different ages, which was usually quite considerable, and most evident in the smaller arteries. In the smallest size group of muscular pulmonary arteries (Figure 3.11) the mean Intima Indices for subjects less than forty years old covered a narrow range 0.03 to 0.07. There was little, if any, change until mid to late fifties by which time the range had extended to almost 0.15 (at the very least a reduction in lumen calibre of 15%). However, the greatest variation was seen in the

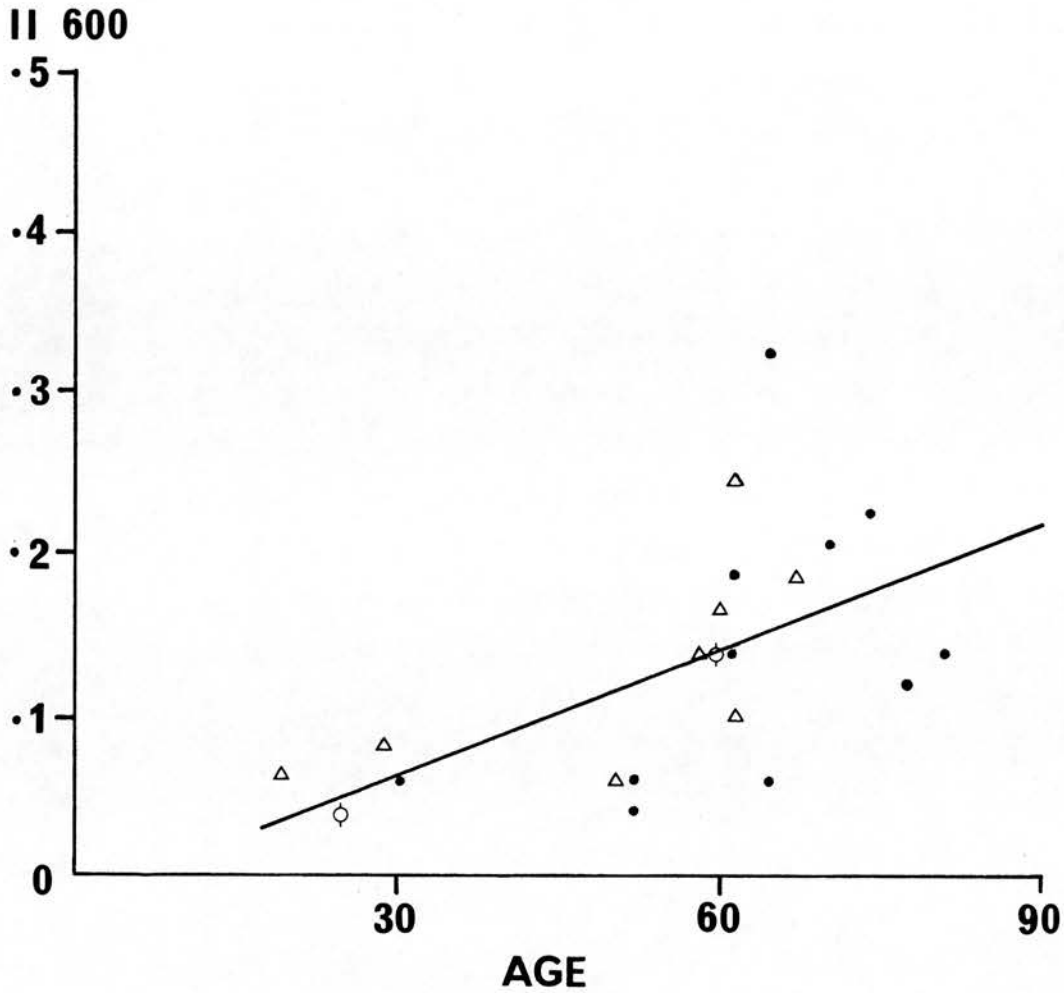


Figure 3.11 The relationship between mean Intima Index 600 and age in the autopsy group.

The lines of best fit are:

Whole group (illustrated)	$y = -0.0180 + 0.00258x$, $r = 0.61$
Non-Smokers (.)	$y = -0.0303 + 0.00268x$, $r = 0.54$
Smokers (△)	$y = 0.0235 \times e^{0.02963x}$, $r = 0.80$

ϕ = Coincident points

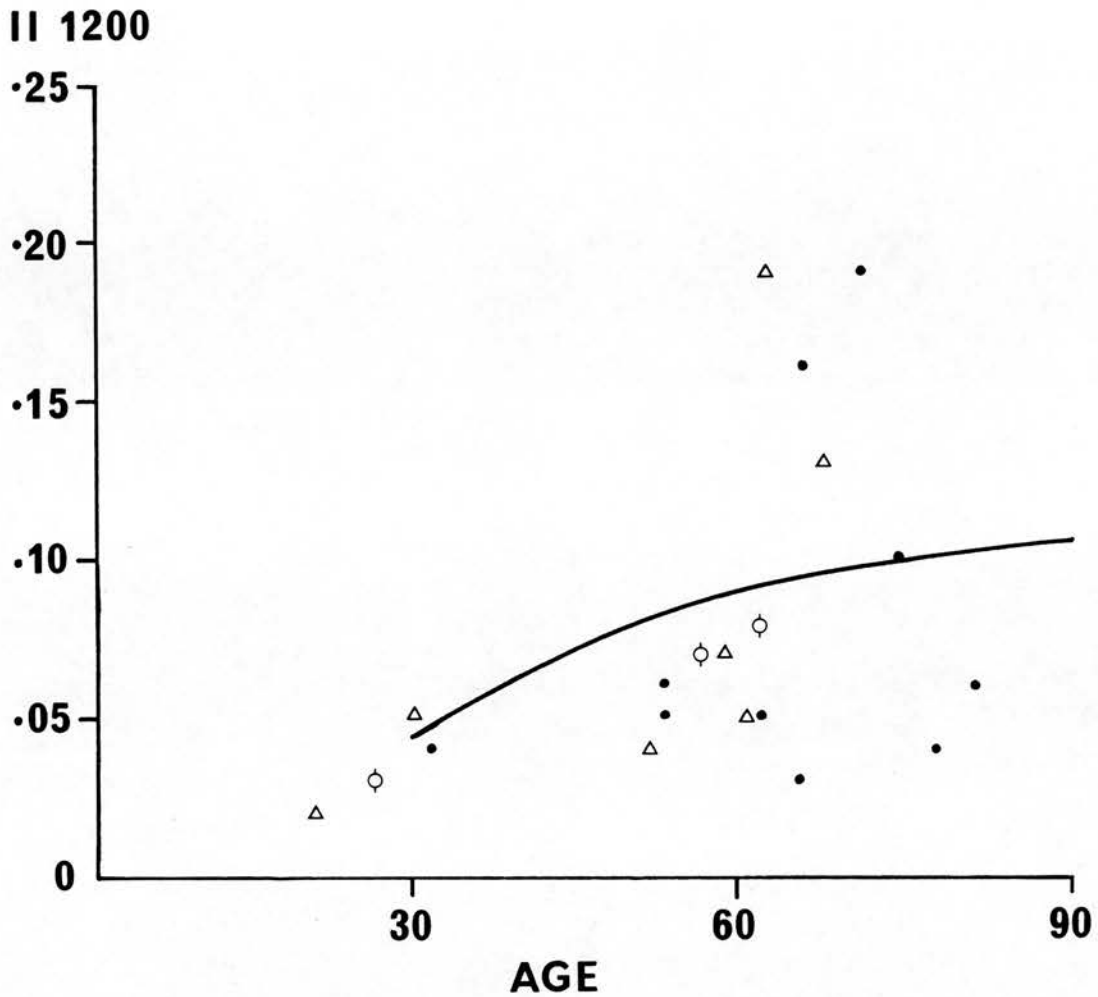


Figure 3.12 The relationship between mean Intima Index 1200 and age in the autopsy group.

The lines of best fit are:

Whole group (illustrated)	$y = -0.1612 + 0.05965.\log_e x$, $r = 0.49$
Non-Smokers (·)	$y = -0.1514 + 0.05588.\log_e x$, $r = 0.39$
Smokers (△)	$y = 1/(52.3784 - 0.66454x)$, $r = 0.71$

ϕ = Coincident points

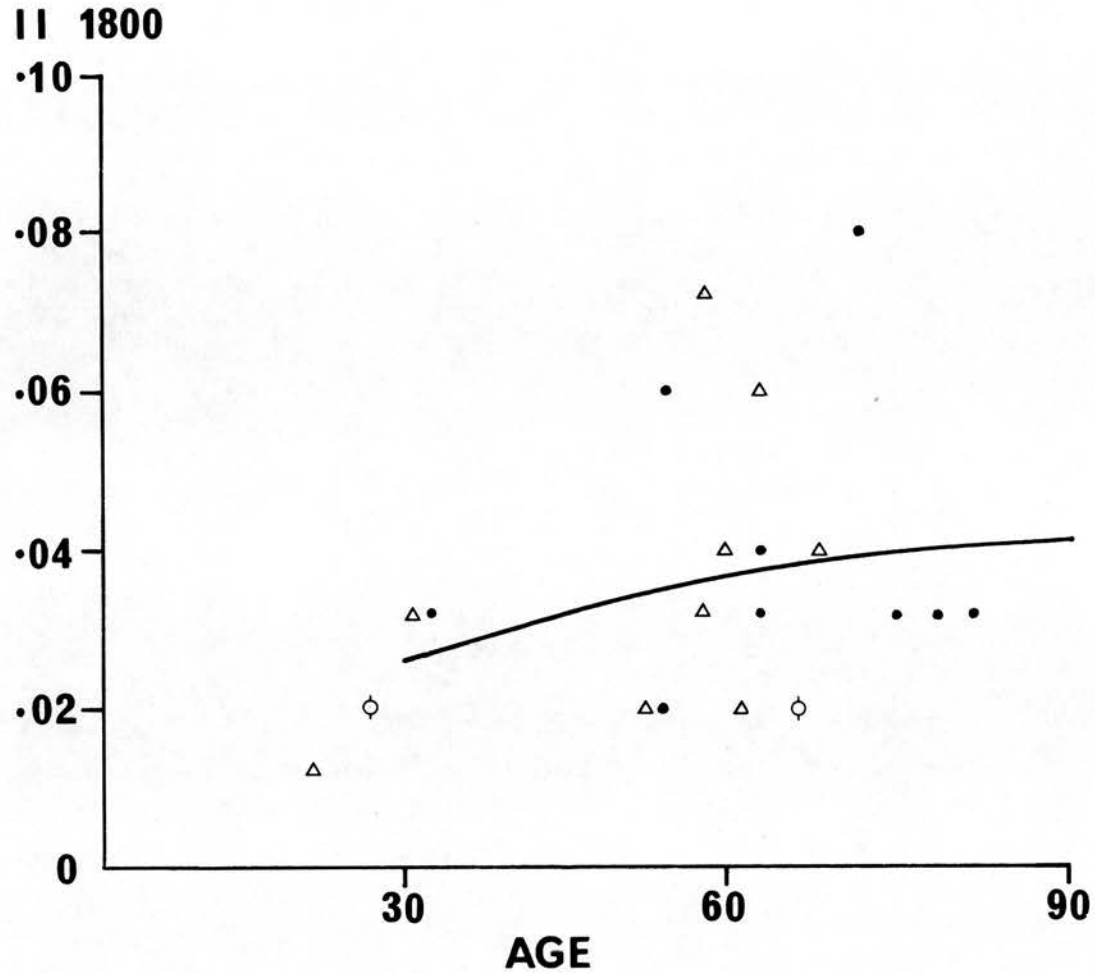


Figure 3.13 The relationship between mean Intima Index 1800 and age in the autopsy group.

The lines of best fit are:

Whole group (illustrated)	$y = 0.0488 - 0.68273/x$	, $r = 0.41$
Non-Smokers (·)	$y = 0.0439 - 0.51553/x$	, $r = 0.24$
Smokers (△)	$y = -0.0606 + 0.02495.\log_e x$,	$r = 0.58$

◊ = Coincident points

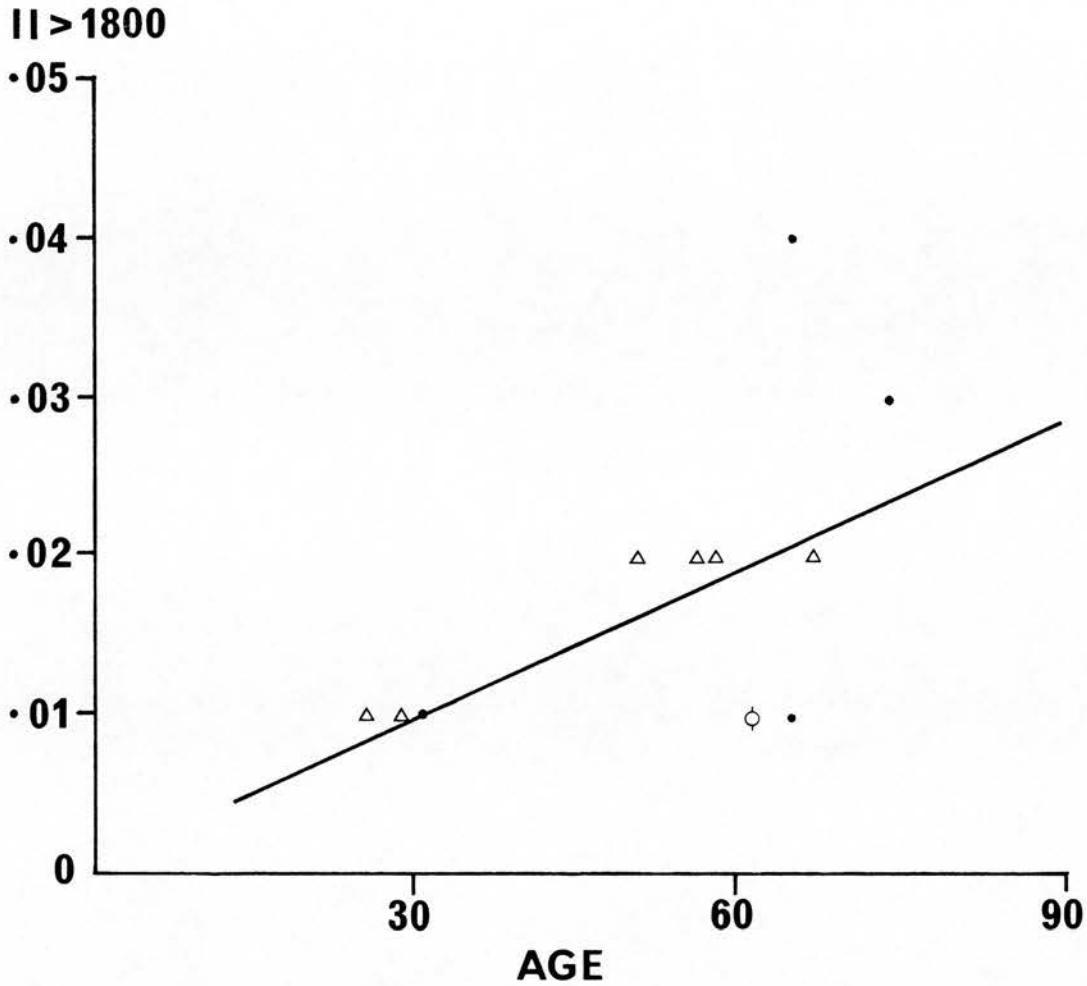


Figure 3.14 The relationship between mean Intima Index >1800 and age in the autopsy group.

The lines of best fit are:

Whole group (illustrated)	y = 0.0005 + 0.00032x, r = 0.53
Non-Smokers (·)	y = -0.0066 + 0.00042x, r = 0.46
Smokers (△)	y = 0.0283 - 0.48218/x, r = 0.97

◊ = Coincident points

age group sixty plus with some individuals showing mean II600 values that were of the same order as those seen in subjects aged forty or less. At the other extreme there were individuals whose muscular pulmonary arteries showed a degree of intimal abnormality that, on average, amounted to a 32% effective lumen reduction (minimum value).

With regard to the II1200 values a similar picture was observed (Figure 3.12), the greatest variation being seen in the sixty plus age group. However, at any age the mean II1200 values (overall range 0.02 to 0.19) were less than the II600 values (overall range 0.03 to 0.32) indicating less severe intimal abnormality with increasing size of artery. This trend continued and became even more marked; in the 1800 size group the maximum mean Intima Index observed was 0.08 (Figure 3.13) whereas in the >1800 size group it was only 0.04 (Figure 3.14).

When the autopsy group was sub-divided by smoking habit, and non-smokers and smokers analysed separately, some very interesting results emerged. For the <u>non-smokers</u> regression of mean II600, II1200, II1800 and II>1800 against age revealed that the lines of best fit were not always linear (see captions to Figures 3.11 - 3.14) but furthermore, none of the slopes of the regression lines was significantly different from zero. This was surprising, especially in the 600 and 1200 size groups, but it is undoubtedly accounted for by the enormous individual variation in the values observed at most ages, but particularly in the sixty plus group (Figure 3.11 and 3.12)

Different results were obtained for the smokers. First of all the relationship which existed between the mean Intima Indices and age was never a linear one (see captions to Figures 3.11 - 3.14). Secondly, in all but one of the size groups, the 1800 group being the exception, the increase in mean Intima Index with increasing age was statistically significant. Starting with the smallest size group the p values were 0.01, 0.05, 0.001 respectively. In pathological terms, however, it is likely that the increase is significant only for the two smallest size groups of arteries (the 600 and 1200). For the >1800 group the increase fom 0.01 to 0.02 (Figure 3.14), although statistically significant, is probably of no functional importance.

In view of the very strong relationship between mean II600 and II1200 and age in the smokers it was decided to investigate the relationship between these measures of intimal abnormality and the amount smoked. The latter was expressed as estimated pack years, this being calculated as follows:

$$\frac{\text{number cigarettes smoked per day}}{20} \times \text{years smoked}$$

Regression of mean II600 against estimated pack years revealed a positive and significant ($p<0.02$) association (Figure 3.15). In the 1200 size group the relationship was also such that the mean Intima Index increased significantly ($p<0.05$) with increasing pack years (Figure 3.16). However, it should be pointed out that this association may reflect an age rather than a smoking effect, age being a very important component of the estimated pack years equation. Furthermore, a comparison of similar aged smokers and

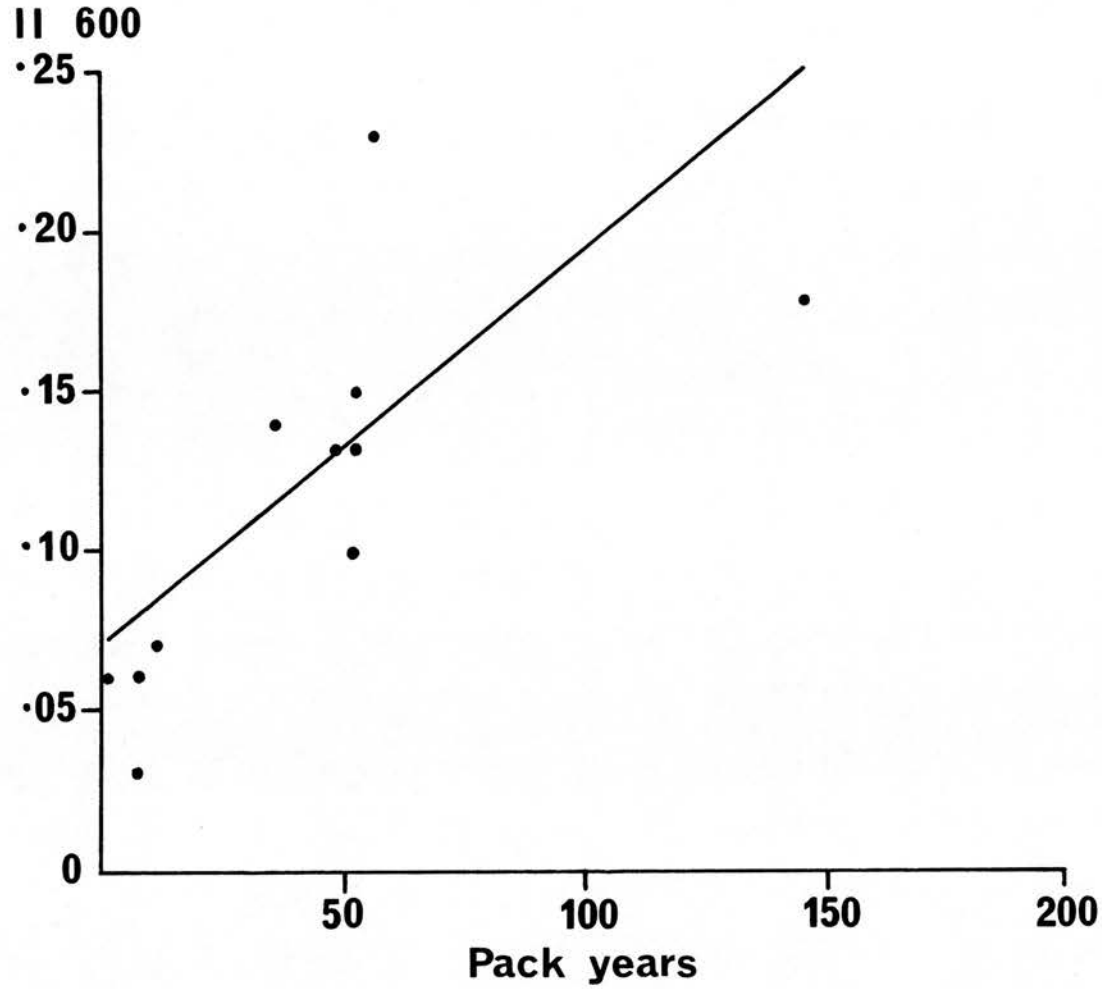


Figure 3.15 The relationship between mean Intima Index 600 and estimated pack years for smokers in the autopsy group.

The line of best fit: $y = 0.0711 + 0.0011x$, $r = 0.71$

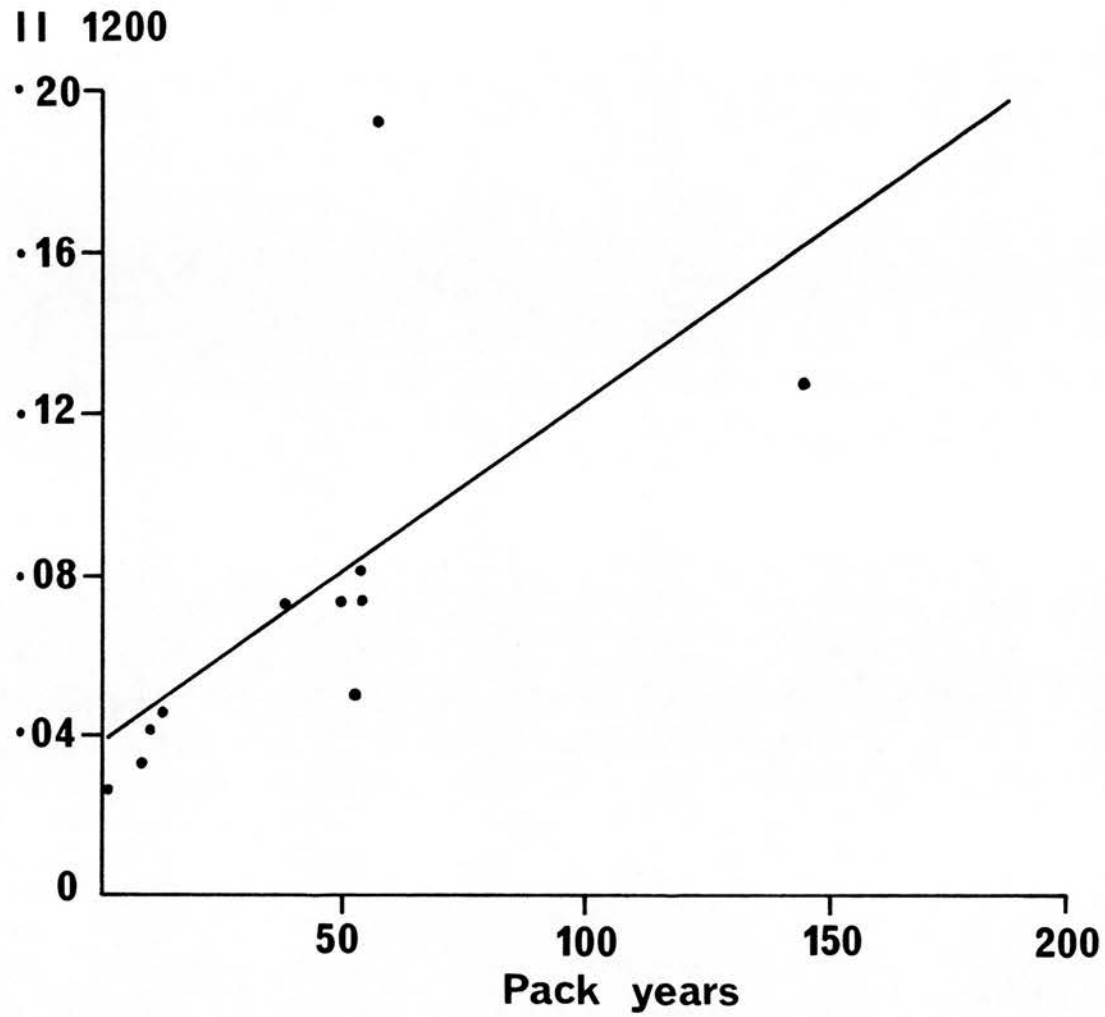


Figure 3.16 The relationship between mean Intima Index 1200 and estimated pack years for smokers in the autopsy group.

The line of best fit: $y = 0.0384 + 0.0008x$, $r = 0.65$

non-smokers in Figures 3.11 and 3.12 reveals that there are no obvious differences between these two groups with respect to intimal abnormality; this in itself suggests that in the smokers it is age rather than smoking that is affecting the intima.

(ii) The resection group

For each subject mean Intima Indices were calculated for muscular pulmonary arteries in the four size groups, and using Minitab these values were plotted and regressed against age, separately identifying males and females and also the different smoking habit groups. In all four size groups of arteries there was absolutely no association between Intima Index and age either for the whole group, for males or females, or for the different smoking habit groups. Figure 3.17 serves as an example to illustrate just one of these points, namely the lack of an age effect on the amount of intimal abnormality in the small muscular pulmonary arteries of both males and females.

Ignoring age, since there was no age effect, the subjects in the resection group were sub-divided first of all by smoking habit and then by sex, and overall mean Intima Indices calculated for arteries in the four size groups. Table 3.3 shows that there were no differences between ex-smokers and smokers in terms of the overall values for mean Intima Index in any of the size groups. However, in all four size groups of arteries, males showed a slightly higher degree of intimal abnormality than females; although this difference was significant in two of the size groups, the 1200 arteries and the >1800 arteries ($p<0.02$ and $p<0.05$ respectively), it is probably of minor functional importance.

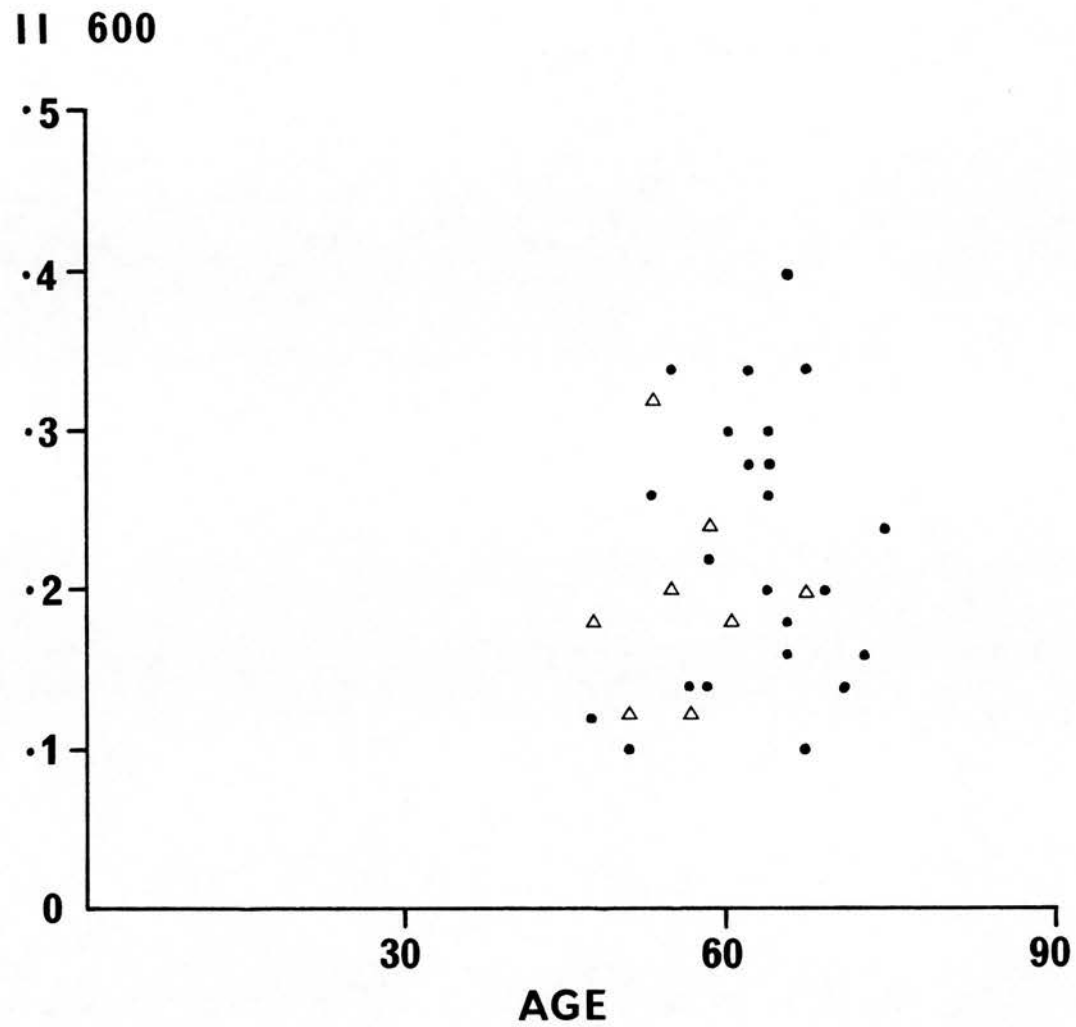


Figure 3.17 The relationship between mean Intima Index 600 and age in the resection group. Males (·) and females (△) separately identified.

Table 3.3 Overall mean Intima Indices for the four size groups of arteries for subjects in the resection group sub-divided by sex and smoking habit.

Sub-group	n	II 600	II 1200	II 1800	II > 1800
Ex-smokers	4*	0.21 (0.08)	0.14 (0.05)	0.06 (0.03)	0.03 (0.006)
Smokers	27+	0.21 (0.08)	0.14 (0.06)	0.07 (0.05)	0.04 (0.02)
Males	23#	0.22 (0.09)	0.15 (0.07)	0.08 (0.05)	0.04 (0.02)
Females	8	0.19 (0.06)	0.11 (0.02)	0.06 (0.02)	0.03 (0.006)

* reduced to 3 in the II > 1800 group

+ reduced to 26 in the II > 1800 group

reduced to 21 in the II > 1800 group

Standard deviations given in brackets

Casting aside the minor male/female differences in degree of intimal abnormality, which was not appropriate to comment upon in the autopsy group, the results for the resection group are at variance with those for the autopsy group, there being no evidence of an age/smoking effect on the intima (result not illustrated). The most fundamental difference, however, between the autopsy and resection groups lay in the Intima Index values observed in the four size groups of arteries. Taking the 600 group as an example, the range of mean II600 values was 0.03 to 0.32 in the autopsy group and 0.09 to 0.40 in the resection group. Although not obviously different the important point is that in the resection group 19 of the 32 subjects showed a mean II600 value of 0.20 or greater whereas in the autopsy group only 3 of the 23 subjects did so. This trend of a higher proportion of subjects with a more severe degree of intimal abnormality in the resection group remained constant throughout the other three size groups of arteries.

3.4.4 Investigation of the Relationship Between Various Clinical and Pathological Factors and the Medial Component of Muscular Pulmonary Arteries

The lack of a relationship between age and smoking habit and the summary data for the media (slope 1500 or MA500, MA1000, MA1500 values) coupled with the individual variability in the values for these parameters (as reported in section 3.4.2) prompted an investigation to determine whether any of the other clinical or

pathological data available correlated with either of the two types of summary data for the media.

For the autopsy group data for the following variables were plotted against the slope 1500 values, and the MA500, MA1000 and MA1500 values:

- height
- % panacinar emphysema
- % centriacinar emphysema
- number centriacinar lesions
- gland:wall ratio
- ratio of weight of left ventricle plus septum to right ventricle
- absolute weight of right ventricle

With one notable exception none of these regressions was significant. The exception was the absolute weight of the right ventricle. It did not correlate with the slope 1500 values but it did show a significant ($p<0.05$) association with the 'predicted' medial area (square root of) values, specifically for the 500μm size point (the MA500 values). This result is illustrated in Figure 3.18. The MA1000 and MA1500 values were not related to the absolute weight of the right ventricle (result not illustrated).

For the resection group data for height were plotted against the summary data for the media, using Minitab.

As with the autopsy group there was no relationship between the height of the patient and the amount of muscle in the walls of the muscular pulmonary arteries.

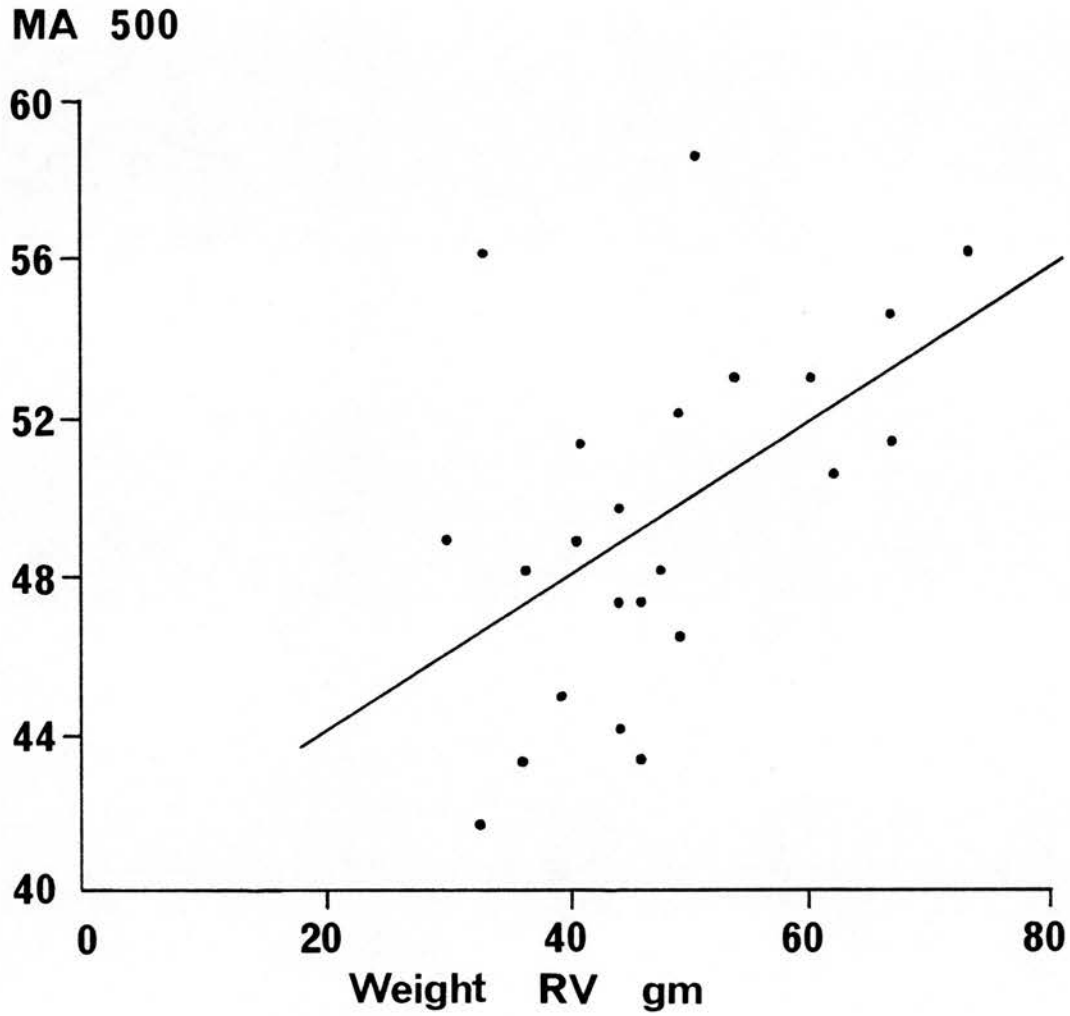


Figure 3.18 The relationship between estimated medial area values for arteries measuring 500μm (length of internal elastic lamina) and absolute weight of right ventricle in subjects in the autopsy group.

Line of best fit: $y = 40.40 + 0.19x$, $r = 0.48$

3.4.5 Summary of the Main Results and Conclusions

1. There was no relationship between the amount of muscle in the walls of muscular pulmonary arteries and the age, sex or smoking habit of subjects in either the autopsy or resection groups.

2. There was considerable individual (inter-subject) variation in the amount of muscle present in any size of artery.

3. In the autopsy group the amount of muscle in the walls of arteries measuring 500μm (length of internal elastic lamina) was positively and significantly correlated with the absolute weight of the right ventricle.

4. In the autopsy group the amount of intimal abnormality in all sizes of artery increased with increasing age.

5. Individual variation was evident in the amount of intimal abnormality present at all ages but was particularly marked in those aged sixty or more.

6. The amount of intimal abnormality decreased with increasing size of artery.

7. In the very small muscular pulmonary arteries the intimal abnormality could amount to an average 32% reduction in lumen calibre in those aged sixty or more.

8. In non-smokers, the increase in intimal abnormality in all sizes of artery with increasing age was not significant due to the enormous individual variation present at all ages.

9. In smokers the amount of intimal abnormality increased with increasing age in all sizes of artery, and significantly so in all but one size group (the 1800 group).

10. In arteries measuring less than 1200μm (length of internal elastic lamina) the amount of intimal abnormality was significantly related to estimated pack years (a measure of amount/duration of smoking). However, this appeared to be largely an age effect.

11. In the resection group there was no evidence of an association between intimal abnormality and either age or smoking habit.

12. Although the range of intimal abnormality was similar in the autopsy and resection groups, the resection group showed a much higher proportion of subjects with a more severe degree of intimal abnormality.

3.5 RESULTS APPENDIX

In addition to the analyses reported in the foregoing Results section several other points were investigated, few of which were directly related to assessment of the effects of age and smoking on the muscular pulmonary arteries. These points were:

1. A comparison of the number of arteries measured in paraffin and glycol methacrylate embedded tissue.

2. Investigation of the relationship between crinkle grade and artery size.

3. A comparison of the crinkle grade of arteries in different lobes.

The first two points were undertaken merely to confirm/refute comments made in Chapter 2 (sections 2.4.11 and 2.4.17 (iii) respectively). Only the third point was of direct relevance to the study of the effects of age and smoking, and necessary for discussion of results from the present study in the light of other workers' findings.

3.5.1 A Comparison of the Number of Arteries Measured in Paraffin and Glycol Methacrylate Embedded Tissue

Mean values for the number of arteries measured were separately calculated for subjects in the autopsy (paraffin embedded) and resection groups (GMA embedded). These values were:

	Mean (standard deviation)
Autopsy group	25.4 (16.1)
Resection group	59.2 (34.3)

3.5.2 The Relationship Between Crinkle Grade and Artery Size

For each subject in both the autopsy and resection groups crinkle grade was plotted and regressed against artery size (total length of internal elastic lamina) using Minitab. The results of this analysis showed that:

1. The relationship between crinkle grade and artery size was not always linear, and

2. Although commonplace, crinkle grade did not always increase with increasing size of artery.

3.5.3 A Comparison of the Crinkle Grade of Arteries from Different Lobes

Using the autopsy group (the only group where tissue samples had been taken from multiple lobes) mean crinkle grades were calculated for the upper and lower lobe arteries of each subject. These 23 sets of mean values were then subjected to a paired t-test which indicated a highly significant ($p<0.0002$) difference between the crinkle grade of upper and lower lobe arteries, those of the upper lobe being much more crinkled (constricted/collapsed).

3.6 DISCUSSION

The Discussion of this chapter is sub-divided into three main sections. The first deals with

- the study groups.

The second and third centre on the muscular pulmonary arteries themselves discussing respectively

- the effects of age and smoking on the media
- the effects of age and smoking on the intima.

The relevant results sections are quoted in brackets throughout.

3.6.1 The Study Groups

It is difficult to obtain material that is suitable for a study such as the present one. Ideally the subjects chosen should have no cardio-pulmonary disease likely to affect the pulmonary circulation, the required clinical data should be sound, reliable smoking histories should be available, and the material itself should be of good quality. When planning the study it was decided to use material resected from patients with small peripheral carcinomas rather than material obtained post-mortem. There were three immediate advantages in doing this. The histological quality of surgical specimens is good and undoubtedly much better than specimens removed post-mortem. Also, the fact that the patients would still be alive would make it possible to obtain all the necessary clinical data and the smoking histories directly. Despite these considerable advantages it was appreciated that the use of

such a group could be strongly criticised on the grounds that the subjects concerned had carcinomas. However, it was intended to include only those with small carcinomas in a peripheral position. Intuitively it was felt that the presence of such lesions would not significantly affect the pulmonary vasculature. Furthermore, the results of a study by Smith & Heath (1980) of age-associated intimal fibrosis, based on resection specimens, had indicated nothing to the contrary, which was encouraging.

While measuring the muscular pulmonary arteries of subjects in the resection group it was noted that quite a large proportion showed extensive and severe intimal fibrosis. This immediately led to a reappraisal of the suitability of the resection group for studying the effects of age and smoking on the intima and media, and resulted in the selection of an additional group for study. There were other reasons for perhaps considering the resection group unsuitable for the present study, non-smokers were rare, and the age range of the patients was narrow, most of them being fifty or older. Since few of the foregoing problems were present in the autopsy group it may seem odd that this group was not the one originally selected for study. The explanation for this lies in the fact that the tissue samples of this group had been embedded in paraffin wax. At the very outset of the study it was uncertain whether paraffin embedded tissue could be used; it was thought that the considerable shrinkage which occurs during the embedding process might produce artefacts in the measurements of pulmonary arteries. Therefore, at the starting date for collecting specimens for the age/smoking study it was decided to use glycol methacrylate embedded tissue. The

study later authenticated the use of paraffin embedded tissue (section 2.4.11).

The only real disadvantage of paraffin compared to glycol methacrylate embedded tissue is the poorer quality of the resulting histological sections. This is borne out by the average numbers of arteries measured per subject in the autopsy and resection groups, more than twice as many being measured in the latter group (section 3.5.1). Nevertheless, in several respects, other than those already mentioned, inclusion of the autopsy group was considered advantageous. To start with the group was similar to the groups used by the majority of other workers to study the effects of age and smoking on the pulmonary arteries (e.g. Hale et al., 1980; Naeye & Dellinger, 1971; Wagenvoort & Wagenvoort, 1965a). This made the comparison of results obtained in the present study with those of other studies considerably easier. However, since the results obtained from the resection group were so interesting it is proposed that the results of both groups be discussed in parallel.

3.6.2 Muscular Pulmonary Arteries: The Effects of Age and Smoking on the Media

Although the principle behind many of the techniques employed in the present study was first described in 1962 (Furuyama), the present study is one of the first of its kind to make full use of a semi-automatic measuring system for obtaining the appropriate measurements. More important it is the first to use these measuring techniques in a study designed solely to establish whether age and smoking affect the medial component of pulmonary arteries and, if

so, to what extent. The importance of such studies should not be underestimated but it appears to have been, to judge by the paucity of reported studies. In order to accurately quantitate alterations in disease states it is necessary to establish the range of values observed in 'undiseased' individuals, and to establish whether there are any differences within the lung itself.

Inevitably, the present study has its limitations insofar as not all arteries were counted and sized, only those cross-sectionally cut. The aims of the study did not include determination of whether age and smoking affect the distribution of arteries by size, a point other workers (e.g. Hale et al., 1980; Semmens, 1970) have investigated, albeit using external diameter as an indicator of artery size with all the inherent disadvantages that this has in both injected and uninjected arteries (as discussed in Chapter 2, sections 2.5.1 and 2.5.3).

Although unable to comment upon differences in size distributions, the present study can comment upon what happens in muscular pulmonary arteries as a result of age and smoking, and it has helped to clarify some of the divergent results obtained by other workers using inappropriate methods of measurement. It has also added important new information (the association between the amount of muscle in the walls of small muscular pulmonary arteries and the absolute weight of the right ventricle discussed on page 203) which came to light as a result of the summary data chosen to describe the muscular pulmonary arteries of a subject. The concept of using the slopes of the regression lines between a function of

medial area and length of internal elastic lamina is not new; it has been used by Niwa (1971) and Suwa & Takahashi (1971) in studies of pulmonary arteries in sudden death of young and apparently healthy subjects, and the arterial (systemic) muscular coat in normal and hypertensive conditions. Similarly, other workers have used these linear regression equations to 'predict' the amount of muscle present in the walls of arteries of a specific size, notably 100μm equivalent external radius (e.g. Suwa & Takahashi, 1971; Yamaki & Tezuka, 1976). However, this type of summary data has not been used in previous studies of the effects of age and smoking on the muscular pulmonary arteries. The latter studies have invariably involved calculation of a mean percentage medial thickness value based either on all arteries measured (Wagenvoort & Wagenvoort, 1965a; Warnock & Kunzmann, 1977a) or on arteries sub-divided into size groups (Hale et al., 1980).

In accordance with Elliott (1964), Simons & Reid (1969), and Wagenvoort & Wagenvoort (1965a) the present study could find no differences in the medial component of pulmonary arteries from males or females (section 3.4.2) or between upper and lower lobes (section 3.4.1). These findings simplify study of the effects of age and smoking since there are no complicating variables to take into account.

This study has shown quite categorically that the amount of muscle in the walls of muscular pulmonary arteries in adult subjects is not affected by ageing (section 3.4.2) or smoking (section 3.4.2). Wagenvoort & Wagenvoort (1965a), whose study group ranged in age from two to 89 years, also concluded that age has no effect

on the media, the latter assessed as a mean percentage medial thickness value. Using similar summary data to Wagenvoort & Wagenvoort (1965a) Semmens (1970), Simons & Reid (1969) and Warnock & Kunzmann (1977a) reported that age does have an effect, in that the muscle layer becomes thicker with advancing years. The reason for this apparent discrepancy is related to the fact that in Wagenvoort & Wagenvoort's study (1965a) the pulmonary arteries were not distended by an injection medium whereas in the other three studies they were. In only one of these three studies (Warnock & Kunzmann, 1977a) did the workers concerned suggest that their findings might be an artefact caused by reduced distensibility of vessels with increasing age, possibly the result of increased intimal fibrosis. It seems more than likely that this is the case, certainly it is the viewpoint adopted by Wagenvoort & Wagenvoort (1979).

There have been very few studies of the effects of smoking on the media, the emphasis generally being placed on elucidating the effects of ageing, often without taking the smoking history of the subject into account, either because it was not thought to do so (Wagenvoort & Wagenvoort, 1965a) or because no such data were available (Simons & Reid, 1969). With regard to the latter point it should be emphasised that, in the present study, there was reliable and fairly extensive information on smoking history available for all subjects in both the autopsy and resection groups.

Other workers (Hale et al., 1980) have concluded that smoking does affect the media of pulmonary arteries. They found an

increased percentage medial thickness in smokers compared to non-smokers, which was evident in all sizes of muscular pulmonary artery up to 500μm in external diameter (the upper limit of those measured). None of the results of the present study (section 3.4.2) support these observations. It is possible that the results of Hale et al. (1980) are an artefact brought about by an increased degree of constriction in the smokers, a factor which, even if very minor, would probably significantly affect the percentage medial thickness measurement by increasing the wall thickness while reducing the external diameter. A more likely explanation, however, is that the smokers and non-smokers chosen for that particular study were not comparable subjects. In describing their study population Hale et al. state -

> "No person had disabling cardio-pulmonary disease, and all were ambulatory prior to death" (Hale et al., 1980)

The very wording suggests that there were study subjects with more than just minimal disease and the probability is that these subjects were smokers. If so, there is the possibility that the smokers selected for study were not a representative sample of smokers in general, and that the observed increased percentage medial thickness was merely a reflection of increased pulmonary disease.

One of the striking findings in the present study was the similarity between the autopsy and resection groups with respect to the media. Neither group showed an age or smoking effect on this vascular component (section 3.4.2), and the range of values observed in both groups was identical (section 3.4.2). This ties in with comments made by Smith & Heath (1980) who reported that, in their series of five resection specimens, there was no evidence of medial

hypertrophy in four and in the fifth a few muscular pulmonary arteries showed but slight medial thickening. On the other hand it has been reported (Wagenvoort & Wagenvoort, 1965b) that bronchial carcinomas may induce medial hypertrophy of the pulmonary arteries of the surrounding lobe. These workers also state that this finding is not simply an artefact caused by an increased degree of constriction; it is real as certified by an increased medial surface area per unit area of lung tissue compared with similar aged controls. Since the latter technique provides a fairly crudely derived estimate of medial area (see section 2.1.2 (iv)) it may be that these results are inaccurate. Assuming that they are accurate, however, a more probable explanation of the apparent discrepancy in the results of these studies is that in Wagenvoort & Wagenvoort's study the 14 study subjects were autopsy specimens whereas in Smith & Heath's study and the present study they were resection specimens. In view of this it is more than likely that the carcinomas in Wagenvoort & Wagenvoort's subjects were more advanced and therefore much more likely to have significantly affected pulmonary function. Another factor which may be relevant here is that chronic bronchitis is commonly seen in patients dying of bronchial carcinomas (Dr David Lamb, personal communication) and the medial hypertrophy observed in Wagenvoort & Wagenvoort's study (1965b) may be related to chronic hypoxia associated with chronic bronchitis. Since Wagenvoort & Wagenvoort do not give any further details of their study subjects it is impossible to determine whether this hypothesis is a sensible one or not.

Some workers studying, for example, age (Warnock & Kunzmann, 1977a) or smoking (Hale et al., 1980) have sub-divided their subjects into broad groups. The main drawback of doing this is that it tends to blur the individual variation evident in the amount of muscle present in muscular pulmonary arteries, as found in the present study (section 3.4.2). This variation, if not actually remarked upon by other workers, is evident from the graphs shown in their published papers (e.g. Hale et al., 1980; Wagenvoort & Wagenvoort, 1965a). It is strange that other workers have played down the aspect of individual variation, for, apart from the lack of an age or smoking effect on the media, it seems that this is the single most important factor to emerge from such studies.

One of the factors it was thought might explain the individual variation in the amount of muscle was the height of the subject (insofar as bigger subjects have bigger lungs) but this was not the case (section 3.4.4). Neither was the amount of muscle related to any of the pathological data available for the lungs of subjects in the autopsy group (section 3.4.4), these data being the percentage of the mid-sagittal slice involved in panacinar and centriacinar emphysema, and the gland:wall ratio of the main bronchus. These latter findings were not unexpected in view of the lack of anything other than minimal disease in the group as a whole. There have been reports (Hale et al., 1980) of associations, specifically in smokers, between percent medial thickness and both small airway disease and centriacinar emphysema. The methods of assessment ('wall thickness') used in Hale et al.'s study are not beyond criticism but a more important criticism of the study is that the

authors seem largely unaware of the fact that the reported associations may be spurious, and that the real cause of the medial hypertrophy may be another factor entirely, one found in association with small airway disease and centriacinar emphysema, such as hypoxia.

Much of the foregoing discussion of the effects of age and smoking on the media seems irrelevant in the light of the observed linear association in the autopsy group between the absolute weight of the right ventricle and the amount of muscle in the walls of small (500μm length of internal elastic lamina) muscular pulmonary arteries. This is one of the most significant findings to emerge from the present study (section 3.4.4). On reflection it is not an unlikely association, given that it is in the small arteries that the major site of vascular resistance is found.

Using measuring techniques similar in theory to the present one, and predicting medial thickness for certain sizes of artery from linear regression equations, other workers have found correlations (not always linear) between the medial thickness of arteries with a radius of 100μm and systolic arterial pressure. This has been shown in a range of systemic arteries (Suwa & Takahashi, 1971) and also in muscular pulmonary arteries in conditions such as ventricular septal defect (Yamaki & Tezuka, 1976), transposition of the great arteries (Yamaki & Tezuka, 1976) and plexogenic pulmonary arteriopathy (Yamaki & Wagenvoort, 1981). The study groups in these three studies either included subjects

with normal pressures (Suwa & Takahashi, 1971) or were composed of subjects all of whom were hypertensive (Yamaki & Tezuka, 1976).

As Suwa & Takahashi point out (1971) the muscular coat of an artery is endowed with a certain tone to counteract the tension exerted on it, and it can regulate the dimensions of the artery lumen. The process does, however, require a certain quantity of physical work which will increase as the blood pressure rises. Because of this it is expected that medial hypertrophy will take place in response to increased work. The results of the three studies mentioned in the preceeding paragraph seem to bear this out. In the present study none of the subjects had pulmonary hypertension or more specifically all had a left ventricle plus septum to right ventricle ratio that was within normal limits (greater than 2.0) as defined by Fulton et al. (1952). Yet even in this group of 'normals' there was a positive structural/ functional correlation. While this correlation is of unquestionable interest and importance it might be a complicating factor in elucidating the effects of disease states on the medial component of pulmonary arteries.

3.6.3 Muscular Pulmonary Arteries: The Effects of Age and Smoking on the Intima

The intima has received less attention that the media in studies of 'undiseased' individuals, which is a reflection of the importance most worker's place, perhaps mistakenly, on medial hypertrophy in disease states, and its assumed link with functional alterations in the pulmonary circulation. However, since small changes in vessel calibre due to intimal thickening would have a

disproportionately large effect on pulmonary vascular resistance it may be that the importance of intimal abnormality has been greatly underestimated.

One of the drawbacks of assessing intimal abnormality is that it rarely affects the entire circumference of an artery to a uniform extent, and it may also be patchy in the sense that not all vessels are affected to the same extent. With regard to studies of the effects of age and smoking on the intima of pulmonary arteries the techniques employed by other workers are crude in comparison to those used in the present study. Crude, not only with respect to the measurement of individual arteries but also with respect to the summary data used to describe a subject. With the latter the most common practice is to simply calculate an overall mean percentage intimal thickness for the arteries of each subject. Since there is a tendency for the smaller arteries to be more affected by intimal abnormality (sections 3.4.1 and 3.4.3), this practice cannot be recommended unless one is certain that the distribution of measured arteries by size is the same in different subjects.

On the topic of size distributions or numbers of arteries, the present study was not designed to count the numbers of arteries affected by intimal fibrosis. Other studies designed to do this have shown that even in older individuals only about 50% of arteries at most are affected (Warnock & Kunzmann, 1977a).

The present study has confirmed previously reported findings that the amount of intimal abnormality increases with increasing age (Warnock & Kunzmann, 1977a; Wagenvoort & Wagenvoort, 1965a;

Wagenvoort & Wagenvoort, 1977) although at any age there is considerable individual variation in the amount of abnormality present, particularly so in older individuals (Wagenvoort & Wagenvoort, 1965a; Wagenvoort & Wagenvoort, 1977). The appropriate results section is 3.4.3. These results refer solely to the autopsy group; there were marked differences between the autopsy and resection groups with regard to the intima, which are discussed later (p210).

Although it has been stated that the extent of intimal abnormality associated with ageing is much less prominent in the larger muscular pulmonary arteries (Semmens, 1970; Wagenvoort & Wagenvoort, 1965a; Warnock & Kunzmann, 1977a) the present study is one of the first to provide an accurate measure of just what that extent is; in those arteries measuring greater than 1200μm length of internal elastic lamina the extent of intimal abnormality never amounts to more than an average reduction in lumen calibre of 10%, even in very old individuals. This is unlikely to be of any functional significance to the pulmonary circulation.

In contrast the extent of intimal abnormality in the very small muscular pulmonary arteries increases markedly with increasing age beyond the age of forty to forty-five. Although there is a very marked individual variation in the mean Intima Index of arteries in this size group (section 3.4.3) the extent of intimal abnormality can amount to as much as a 32% reduction in lumen calibre in those aged sixty or more. There are a number of separate points that need to be made here. Firstly, these results corroborate the findings of

Wagenvoort & Wagenvoort (1965a) and Hale et al. (1980) who, in studies of the effects of age and smoking on the pulmonary arteries, found mean percentage intimal thickness (percentage of internal diameter) values in the order of 20%. Secondly, in some subjects, it seems unlikely that a mean effective reduction in lumen calibre of 32% in the very small arteries would not cause some functional alterations in the pulmonary circulation as a whole, especially when one cosiders that the value of 32% is a minimum possible value, the Intima Index being a theoretical measure of intimal abnormality or lumen reduction, which is independent of the degree of active constriction present in the artery. The third point is that the present study has established the range of values one would expect to see in 'undiseased' individuals in the very small muscular pulmonary arteries which, as far as intimal abnormality is concerned, seem to be the most reactive. The fact that this range is quite extensive, particularly in older individuals, will undoubtedly affect the assessment and interpretation of intimal abnormality in disease states.

Although none of the aforementioned age effects was statistically significant in the group of non-smokers (section 3.4.3) this was undoubtedly due to the huge individual variation already discussed. The picture in the smokers was different; the amount of intimal abnormality increased with increasing age in all sizes of artery (section 3.4.3) although it must be said that in arteries measuring >1200μm length of internal elastic lamina the increase is unlikely to be of any functional importance.

It proved somewhat difficult to establish whether or not smoking has a direct effect on the intima.

There was an association in the small and medium sized muscular pulmonary arteries (those measuring <1200μm length of internal elastic lamina) between estimated pack years and extent of intimal abnormality. Such an association has not been commented upon before since the policy of most workers has simply been to sub-divide their subjects into broad smoking groups, e.g. smokers and non-smokers (Hale et al., 1980; Naeye & Dellinger, 1971). Only Auerbach et al. (1963) have attempted to further sub-divide smokers on the basis of the number of packs smoked per day. It is unfortunate that they did not assess the media and intima separately because they found that the overall thickness of the walls of small vessels increased with increasing amount smoked.

The fact that estimated pack years has a strong age component is a complicating factor in this issue. In the smokers in the present study it appeared that it was age rather than smoking that was important since the amount of intimal abnormality in the smokers was not markedly different from that seen in the non-smokers (section 3.4.3). However, the age ranges of the two groups were different and it would be useful to measure the arteries of some old (seventy or older) smokers. It is intended that this be done shortly, and hopefully the results will help clarify whether smoking has an effect on the intima.

The contributions made by the present study do not end with the production of very accurate information on the amount and range of intimal abnormality seen in a group of 'undiseased' individuals of different ages and smoking habits. Indeed it is considered that one of the most useful contributions it has made is to dispel a long-held belief on the part of many workers (e.g. Hale et al., 1980; Wagenvoort & Wagenvoort, 1965a) that intimal abnormality shows a predilection for the upper lobes of the lung not only in 'undiseased' individuals but also in those with mitral valve disease (Wagenvoort, 1975). Admittedly it has puzzled workers why this should be so; Wagenvoort in particular has stated-

> "It is hard to explain this unexpected finding" (Wagenvoort & Wagenvoort, 1965a).

A number of theories have been put forward but the most commonly held opinion is that it results from differences in blood flow in the upper and lower lobes of the lung, the reduced blood flow in the apex causing an increased tendency to thrombosis in periods of lung infections etc. (Wagenvoort & Wagenvoort, 1965a; Wagenvoort & Wagenvoort, 1979). The present study has shown that there is no need for these complex theories; quite simply there are no differences between lobes in 'undiseased' persons, in terms of intimal abnormality, but there are functional differences in arteries from different lobes, those from the upper lobes tending to be more constricted/collapsed than those from the lower lobes (section 3.5.3). It is this latter factor, coupled with a measuring technique that is not independent of the degree of collapse or constriction present, that has resulted in the conclusions drawn by other workers that the percent intimal thickness is greater in

arteries from the upper lobes (e.g. Hale et al., 1980; Wagenvoort & Wagenvoort, 1965a). The importance of appropriate measuring techniques becomes ever more firmly underlined when situations such as these come to light.

To end this Discussion it seems fitting to discuss the differences observed between the autopsy and resection groups with regard to intimal abnormality since it was these differences that primarily led to a virtual abandonment of the resection group as a control population for studying the effects of age and smoking on the muscular pulmonary arteries. If one considers the autopsy group as a group of 'normals' then the same cannot be said about the resection group. For a start, there was no age or smoking effect on the intima (section 3.4.3), and although the upper limit of intimal abnormality in all size groups of artery was not much higher in the resection group than in the autopsy group (section 3.4.3) it was very noticeable that a much higher proportion of subjects showed mean Intima Indices approaching the upper limits. Furthermore, in the autopsy group the intimal thickening was most marked in the small and medium sized muscular pulmonary arteries whereas in the resection group larger arteries were often affected as well (although still to a lesser extent than the smaller ones). These findings support comments made by Wagenvoort & Wagenvoort (1965b) in their study of the pulmonary vasculature in cases of bronchial carcinoma. Overall, the results suggest that, superimposed on the effects of age and smoking, there is a degree of intimal fibrosis which results, either directly or indirectly, from the bronchial carcinoma itself. It may be that the amount of intimal abnormality

seen in the resection specimens is associated with either the size or position of the carcinoma and it is intended that this be investigated in the very near future.

FUTURE WORK

When planning fairly long-term studies there is always a tendency to be overambitious with regard to what can be achieved in the time available. With the present study it was intended that the measuring techniques be applied to studies of the muscular pulmonary arteries in disease states. However, the development of these new measuring techniques and their validation was time-consuming, especially the investigation of the effects of different tissue preparation techniques on the measurements obtained. As a result the study was limited to including only the effects of age and smoking on the pulmonary arteries and even here further work is required. In particular there is a need to measure the muscular pulmonary arteries of some smokers aged over seventy to clarify whether or not smoking has a direct effect on the intima. Another area which requires further investigation is the extent of intimal abnormality seen in the resection group, a factor which was largely responsible for making the group unsuitable for a study of the effects of age and smoking. As a starting point it is intended that the extent of intimal abnormality in this group be related to the size, site and type of carcinoma present; any further research will obviously be dependent on the outcome of this initial investigation.

Once these two areas have been examined the measuring techniques developed in this study will be applied to disease states. At the Institute of Occupational Medicine much of the on-going research is directed towards occupational lung diseases such as pneumoconiosis; in the last ten years two major autopsy studies

of coalworkers have been carried out (Davis et al., 1979; Ruckley et al., 1981), the latter of which included an investigation of the relationship between right ventricular hypertrophy and disease present in the lung (Fernie et al., 1983). A logical follow on would be to quantitate the vascular changes present in coalworkers and to investigate their relationship with the presence of right ventricular hypertrophy and disease in the lung. However, in coalworkers the situation with regard to lung disease is complex. They may have pneumoconiosis, itself a possible cause of pulmonary hypertension and right ventricular hypertrophy; frequently, however, chronic airflow obstruction due to airways disease and emphysema is also present, and it is this condition which is the commonest cause of right ventricular hypertrophy. In such cases it is difficult to determine whether the right sided heart disease and accompanying vascular changes are the result of the occupational lung disease or the airways disease and emphysema, which may not be associated with the dusty occupation. Because of this the next major step in the present research programme will be the quantitation of vascular changes in non-miners with chronic obstructive lung disease (with and without emphysema) and the relationship between the vascular measurements and right sided heart disease. Since a great deal of attention is centred on such subjects the results will be of particular interest. Material from a group of subjects suitable for this study is already available and for all subjects there is extensive data on clinical history, and measurements of respiratory function, pulmonary arterial pressures, and partial pressures of O_2 and CO_2.

It is envisaged that many new problems will arise when the measuring techniques are applied to the aforementioned disease states. With emphysema, for example, one would expect a loss of vessels as the disease becomes more extensive; therefore, the number of arteries present will become important in the interpretation of the vascular measurements. Unfortunately, assessment of numbers of arteries will not be straightforward; differences in size of subject are reflected in variable numbers of arteries per unit area of lung tissue. Methods of coping with problems such as these are currently under consideration.

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A New Method for Quantitating the Medial Component of Pulmonary Arteries

The Measurements

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• **Current methods for quantitating the media of pulmonary arteries are inadequate in that either they produce measurements of the media and artery size that are affected by vasoconstriction, or they are complicated to use. We developed a method that overcomes these problems. It produces measurements of medial area and the artery size is expressed in terms of the total length of the internal elastic lamina. The measurements are obtained directly from histologic sections using a light microscope with a camera lucida attachment in conjunction with a microcomputer linked to a digitizing board. Repeatability of the measurements is excellent but it is essential to digitize at a magnification at which crinkles in the internal elastic lamina are clearly visible. Arteries that are considered to be digitizable are representative of the total muscular pulmonary artery population.**

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For the past 30 years, researchers have been quantitating the structural components of pulmonary blood vessels, particularly the media of muscular pulmonary arteries, with an overall aim of establishing the response of these vessels in various disease states.

Of the many techniques that have been used to assess medial hypertrophy in both pulmonary and systemic arteries, the most common have undoubtedly been the "wall thickness" methods. These methods were first introduced in 1929 in a study of the systemic vasculature, in which the vessel wall thickness was expressed as a ratio of the lumen diameter.[1] Since then, other indices of medial hypertrophy have been derived. It has been assessed simply as medial thickness,[2,3] although most researchers have tried to relate medial thickness to some indicator of vessel size by expressing it as a ratio of the vessel lumen,[4,5] as the external diameter (the distance between diametrically opposed points on the external elastic lamina),[6] or, more commonly, as a percentage of these values, particularly the external diameter.[7-11] Such studies have been criticized[12] on the grounds that measurements of vessel caliber and wall thickness are greatly affected by the degree of vasoconstriction or postmortem collapse of the vessels, which vary considerably.[13] More accurate attempts at measuring the medial layer of arteries have used medial area measurements derived indirectly by mathematical calculation from micrometer measurements[7,14-16] or directly by planimetry.[2,3,17-24] Throughout, however, there has remained the problem of finding a reliable indicator of vessel size to which the measurements could be related. With respect to the pulmonary vasculature, some workers have attempted to overcome the problems associated with diameter by distending the arteries with an injected medium.[15,25-29] Other researchers have continued to work with uninjected material and have tried to define size by the position relative to the accompanying airway.[30] Medial area measurements have also been set in relation to the areas of histologic sections[14] and intimal nuclei,[19] values which are not affected by dilation or constriction of the vessels. Perhaps the most readily understood methods, however, are those in which vessel size is expressed in terms of the length of the internal elastic lamina. Although the use of the length of the internal elastic lamina has been reported in several studies of both the systemic[17,18,22] and pulmonary vasculature,[21,23,24,31,32] it is not a commonly used indicator of vessel size. The reasons for this are almost certainly related to the difficulty of measuring the length of the internal elastic lamina in uninjected material.

The present report describes a digitizing method for measuring various indices of muscular pulmonary arteries, which include the length of the internal elastic lamina. The repeatability of the measurements is discussed. In the assessment of the media and size of arteries, the medial area and length of the internal elastic

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lamina are compared with other commonly used measurements and their relative merits are discussed.

MATERIALS AND METHODS

Subjects

Seven subjects were included in this study. Details of the relevant pathology and lung preparation carried out on specimens from these seven subjects are given in Table 1.

Table 1.—Details of Relevant Pathology and Lung Preparation for Seven Subjects*

Subject No./Age, yr/Sex	Smoking History	Cardiopulmonary Pathology	Pulmonary Artery Injection
1/56/M	Smoker	COLD, marked RVH	One lung only
2/55/M	Smoker	COLD, no RVH	One lung only
3/67/M	Smoker	COLD, marked RVH	One lung only
4/64/M	Unknown	Rheumatic heart disease	One lung only
5/59/M	Smoker	Normal	No
6/67/M	Unknown	Atrial septal defect	No
7/24/M	Unknown	Pulmonary sequestration	No

*COLD indicates chronic obstructive lung disease; RVH, right ventricular hypertrophy.

Injection of Pulmonary Arteries

At autopsy, both lungs were removed intact and one of them was warmed by immersion in water at 37 °C. A cannula was then tied into the main pulmonary artery and a barium-gelatin mixture at a temperature of 60 °C[33] was injected at a pressure in excess of 100 cm H_2O for several minutes. Hypertensive pressures established by other researchers were used[25,33,34] to overcome postmortem contraction and to produce complete distention of the arteries and arterioles. When the injection was complete, the main pulmonary artery was ligated. The other lung was left uninjected and both lungs were then inflated.

Inflation of Lungs

Lungs were inflated by instilling formaldehyde solution into the main bronchus; they were then allowed to continue fixing for at least one week in a basin of formaldehyde solution that was covered to prevent drying.

Preparation of Histologic Sections

Lungs were sliced at 1-cm intervals in the sagittal plane and representative tissue blocks, measuring approximately 2.5 × 2.5 × 1.3 cm, were taken. In general, six blocks were taken from each of the upper lobes and either the lower lobes or lower and middle lobes. Tissue blocks were embedded in paraffin and sectioned at 5 μm. Weigert's elastic stain with a van Gieson counterstain or Miller's elastic stain were used.

Method of Measurement

All of the measurements were recorded with a digitizing system that was composed of a digitizing board with an electronic cursor, together with a microcomputer and a printer.

Measurements of pulmonary arteries were obtained directly from histologic sections by virtue of a camera lucida attachment to the light microscope, with the attachment oriented so that it overhung the digitizing board. This enabled the pinpoint of light emitted from the cursor on the digitizing board to be superimposed on the image seen. Before commencing measurement, the digitizing board was calibrated to the light microscope; this was effected by assigning each of the four buttons on the cursor to a specific lens objective on the microscope. The various measurements of each artery, determined by the program that was used, were recorded by means of the operator moving the pinpoint of light to or around the specified parts of the artery. This was achieved by moving the cursor over the digitizing board while depressing the appropriate button. All coordinates "activated" on the digitizing board were recorded and passed to the microcomputer, which translated the information into distances between points, lengths, or areas.

Measurements may also be obtained from photographs of pulmonary arteries by taping them to the digitizing board, specifying the magnification, and drawing around the various elements with a cursor fitted with a cross hair in place of a light-emitting source.

All of the measurements were printed directly and/or stored on tape for analysis at the operator's convenience.

Two programs were used for measuring arteries. Program 1 was designed to record the following values of muscular pulmonary arteries that satisfied the criteria of being cut in good cross sections and having a well-defined internal elastic lamina around the major part of their wall. The values measured by the observer were as follows: the two external diameters at right angles (D1 and D2); the four corresponding medial thicknesses (M1, M2, M3, and M4); the circumference of the lumen (LC); and the total lengths of the internal elastic lamina (IEL) and the external elastic lamina (EEL). The luminal area (LA), intimal area (IA), and medial area (MA) were measured by computer (Fig 1).

A well-defined internal elastic lamina was of prime importance as the length of the internal elastic lamina was to be used as an indicator of vessel size. If its outline could not be clearly seen around at least seven eighths of any arterial wall, then that artery was not measured. The external elastic lamina did not have to satisfy such stringent criteria as its length was considered a nonessential measurement, with accurate delineation of the media being more important.

Arteries that satisfied these criteria and that were measured using program 1 were termed digitizable.

Program 2 enabled more limited measurements to be obtained for all muscular pulmonary arteries, including those arteries that were considered digitizable and the remaining arteries. Each artery was assigned to a specific class depending on whether it was considered digitizable (ie, measurable using program 1) or not, and also according to the angle of cut, and whether there was a good cross section. For an artery that was cut in a good cross section, the measurements that were made were as follows (Fig 2, left): the two external diameters at right angles (D1A and D2A); the two internal diameters at right angles (D1B and D2B); and the two luminal diameters at right angles (D1C and D2C).

Medial and intimal thicknesses were calculated by subtraction, eg, D1A − D1B and D1B − D1C, respectively. For arteries that were not cut in a good cross section, measurements were made on only the short axis of the artery at the point where it was widest in the plane perpendicular to its long axis (Fig 2, right).

The measurement of diameters or wall thicknesses using these two programs was facilitated by the use of an eyepiece graticule with a cross hair, with the measurements being made at the points where the cross hair intersected the lumen-intima interface, the internal elastic lamina, or the external elastic lamina. This eliminated the need for a subjective decision on where to take these measurements, resulting in greater accuracy and repeatability of the measurements that were obtained.

In addition to these two programs, a series of standard statistical programs was available for analysis of the data collected. All of the programs were written in BASIC.

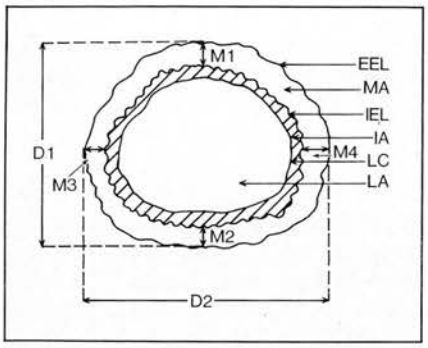

Fig 1.—Diagrammatic representation of a muscular pulmonary artery indicating the structural components and measurements obtained using program 1. D1 and D2 indicate right-angle external diameters; M1, M2, M3, and M4, corresponding medial thicknesses; EEL, external elastic lamina; IEL, internal elastic lamina; MA, medial area; IA, intimal area; LA, luminal area; and LC, luminal circumference.

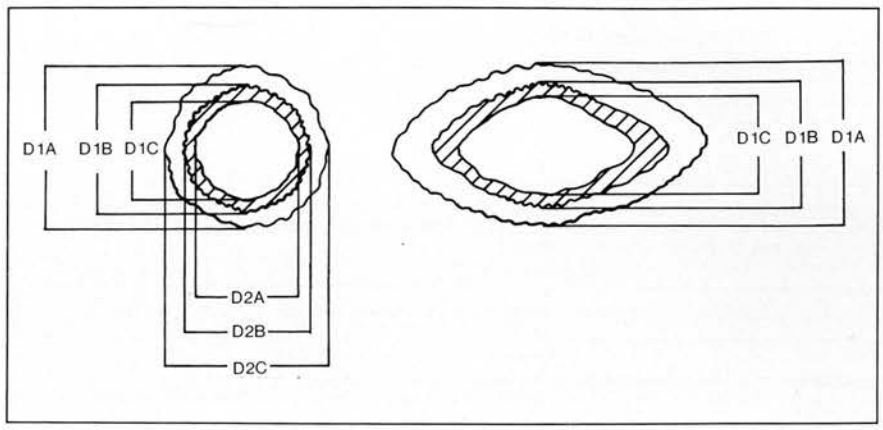

Fig 2.—Diagrammatic representation of muscular pulmonary arteries cut in cross section (left) or otherwise (right), and measurements made using program 2. D1A and D2A indicate right-angle external diameters; D1B and D2B, right-angle internal diameters; and D1C and D2C, right-angle luminal diameters.

Repeatability of Measurements

Fifteen muscular pulmonary arteries were selected from the histologic sections of five subjects (Nos. 1, 4, 5, 6, and 7, Table 1) whose pulmonary vasculature ranged from normal to that associated with chronic obstructive lung disease, rheumatic heart disease, atrial septal defect, and pulmonary sequestration. These arteries were chosen for several reasons. They covered a wide range of abnormality, some possessed features that might affect the repeatability of measurements, eg, very thin media or extremely crinkled elastic laminae, and some were borderline in terms of being considered digitizable. Both injected and uninjected arteries were included. The features that were possessed by the 15 arteries are commonly seen in pulmonary arteries in both the normal and diseased states.

Each artery was digitized three times in succession using program 1 and a mean value was calculated for each criterion that was measured. All 15 arteries were then digitized on three more occasions, with each occasion separated by a minimum period of six weeks.

A subgroup of ten arteries was additionally digitized at different magnifications.

Repeatability of Selection of Arteries Considered Digitizable

As has already been described, a muscular pulmonary artery was considered digitizable if it was cut in a good cross section and showed a well-defined internal elastic lamina around the major part of its wall. To test the stringency of these criteria, histologic sections from four subjects were scanned on three occasions, each separated by a minimum period of four weeks, and the microscope stage coordinates (Vernier scale readings) of each artery that was considered digitizable were recorded. The four subjects were those from whom injected and uninjected material was available. These subjects were chosen because their pulmonary vasculature showed a wide range of abnormality and it was considered important to determine whether the severity of disease affected the selection of arteries that were considered digitizable.

Comparison of Digitizable Arteries With the Total Population

Histologic sections from the same four subjects were scanned and all of the muscular pulmonary arteries were identified. Measurements of each artery were made according to program 2.

RESULTS

Repeatability of Measurements

For each of the 15 muscular pulmonary arteries, the mean values of all of the measurements were calculated from those obtained at three consecutive digitizations. The measurements comprised diameters 1 and 2, and the average medial thickness (MT), luminal area and circumference, total length of internal elastic lamina, medial area, and total length of external elastic lamina. The intimal area value is not included in this article but will be reported on later.

Actual values for each measurement/artery were considered unimportant and are not quoted. For each measurement/artery the maximum percent deviation from the mean was calculated. In Table 2 it can be seen that the values that were obtained for all 15 arteries on consecutive digitizations mostly lay within 2% of the mean value and a high proportion were within 1%. This was evident for all measurements with the notable exception of average medial thickness, where deviations of as much as 10% were observed.

Using the mean values that were obtained from the initial three consecutive digitizations as a baseline, the long-term repeatability of the measurements was investigated. On each of three further digitizations the percent deviation from the baseline value was calculated for each measurement/artery. Table 3 shows the maximum percent deviation that was observed during the three independent digitizing sessions for each measurement/artery, together with the notable features of each artery, if any. Of the 120 values that are illustrated, 93 showed a maximum deviation of less than 5% from the baseline value. Poor repeatability of the medial thickness measurement, which was not unexpected, accounted for 13 of the remaining 27 values. In general, poor repeatability (>5% deviation) of a criterion other than medial thickness in any artery was linked to its structure, eg, a very thin media or very crinkled elastic laminae.

The effect of magnification on the measurements that were obtained for each criterion was investigated in a subgroup of ten muscular pulmonary arteries that fulfilled the requirement of being digitizable at a minimum of three of four magnifications—×4,

Table 2.—Number of Arteries With Deviations From the Mean*

Maximum Deviation From Mean, %	Criterion							
	D1	D2	MT	LA	LC	IEL	MA	EEL
>1	2	0	14	0	1	6	4	4
>2	0	0	14	0	0	2	1	3

*Measurements were obtained at three consecutive digitizations. D1 and D2 indicate right-angle external diameters; MT, medial thickness; LA, lumina area; LC, luminal circumference; IEL, internal elastic lamina; MA, medial area; and EEL, external elastic lamina.

Table 3.—Long-term Repeatability of Measurements of 15 Arteries*

Artery No.	Criterion								Notable Features
	D1	D2	MT	LA	LC	IEL	MA	EEL	
1†	0.9	1.4	10.0	2.9	1.1	1.1	8.3	1.1	Very thin media
2†	1.3	1.2	9.8	0.7	0.2	0.8	2.1	2.4	. . .
3†	0.9	0.4	8.7	0.9	1.1	1.2	6.0	0.2	Very thin media
4†	0.9	0.8	14.6	0.7	0.4	0.5	5.2	0.5	Very thin media
5	1.1	1.1	16.3	0.3	1.9	1.6	5.5	2.6	. . .
6	0.2	1.3	8.3	1.3	1.1	1.7	0.7	0.9	. . .
7	1.3	4.4	9.3	0.9	1.5	3.7	1.9	1.6	. . .
8	0.9	1.4	21.1	1.4	0.5	4.0	3.0	5.3	Partly ill-defined, crinkled elastic laminae
9	2.3	1.5	2.2	2.6	2.2	9.7	0.9	2.1	Partly ill-defined, crinkled elastic laminae
10	0.8	1.4	8.5	1.5	1.3	4.9	1.3	1.9	. . .
11	3.4	1.0	10.7	0.8	1.5	2.5	2.2	1.3	. . .
12	0.6	0.4	5.4	0.6	1.2	3.7	0.3	3.7	Very crinkled elastic laminae
13	1.6	1.8	4.8	1.8	3.1	5.3	2.2	5.2	Very crinkled elastic laminae
14	3.7	2.7	21.9	8.5	6.1	6.3	10.0	8.3	Very small artery
15	2.8	1.6	17.7	1.5	0.7	1.6	2.7	7.1	Partly ill-defined elastic laminae

*Values are expressed as the maximum percentage deviation from the baseline value. D1 and D2 indicate right-angle external diameters; MT, medial thickness; LA, luminal area; LC, luminal circumference; IEL, internal elastic lamina; MA, medial area; and EEL, external elastic lamina.
†Injected artery.

×10, ×20, and ×40. The data are expressed as the ratios measurement at specified magnification/measurement at lowest magnification, and are arranged in order of increasing magnification (Table 4). Thus, a ratio of 1 throughout for any criterion would indicate that that particular measurement was unaffected by magnification.

As expected, the speed of obtaining the measurements decreased with increasing magnification. With regard to the measurements themselves, the results illustrated in Table 4 suggest that the diameter (D1 and D2) and area (LA and MA) measurements are generally less affected by magnification than the length (LC and particularly IEL and EEL) measurements. This was most obvious in arteries that showed marked crenation of the elastic laminae (Nos. 8, 9, and 13) and has important implications if the total length of the internal elastic lamina is to be used as an indicator of artery size. The low ratios of the medial thickness measurement in the majority of arteries is explained by gross inaccuracies in the measurement of this value at low magnification.

The conclusions to be drawn from this aspect of the study are that it is possible to increase the speed of obtaining the measurements by digitizing arteries at low magnification. However, in some arteries, depending on their structure, this increased speed may come at the expense of accuracy of the measurements that are obtained.

Repeatability of Selection of Arteries Considered Digitizable

The histologic sections from four subjects (Nos. 1, 2, 3, and 4 in Table 1) were scanned on three separate occasions and each muscular pulmonary artery that was considered digitizable was identified by the microscope stage coordinates at which it lay in the center of the field of view. Table 5 describes the results, which show a high level of consistency in the selection of arteries that are considered digitizable, thus indicating that the criteria for digitizability are adequately stringent. It was additionally encouraging to find that these criteria were unaffected by the severity of the disease that was present, as the results for subject 4, whose disease was the worst in terms of arterial intimal changes, showed a level of repeatability that was certainly no lower than the level seen in the other three subjects.

Comparison of Digitizable Arteries With the Total Population

It was observed (Table 5) that the number of muscular pulmonary arteries that were considered digitizable was very small in some subjects; the main reason for arteries not being considered digitizable was that they were not cut in good cross sections. This gave rise to the question of whether the digitizable arteries could truly be considered representative of the total muscular pulmonary artery population in terms of the amount of medial muscle present in an artery of a given size. As measurements of undigitizable arteries were necessarily limited to wall thickness and diameter, the comparison of digitizable arteries with the total population was based on these criteria.

Because the majority of arteries were not cut in good cross section, the short diameter was used as the indicator of size. The muscular pulmonary arteries were subdivided by external diameter (D1A) into six groups, and the mean medial thickness (D1A − D1B) and the percent medial thickness were calculated for each size group for both digitizable arteries and the total population using the following formula: $[(D1A - D1B)/D1A] \times (100/1)$. The results for all four subjects showed similar trends, and only those of subject 1 are illustrated (Table 6). The data presented in Table 6 indicate that the digitizable arteries compose a small proportion of the total population with none or few of the arteries being considered digitizable in some size groups. Overall, there was a tendency for a greater propor-

tion of the larger arteries to be considered digitizable.

In terms of mean percent medial thickness values, there appeared to be no striking differences between digitizable arteries and the total population in any of the size groups. Statistical testing of the significance of the difference between these mean values was hampered in some size groups by an insufficient number of digitizable arteries and in other groups by an unbalanced distribution of arteries between the digitizable and total population groups. In those size groups in which it was possible to carry out a Student's t test, only one significant difference between digitizable arteries and the total population was found (subject 2, uninjected lung, size group 200 to 299 μm).

Table 4.—Data for Criteria in Ten Arteries Digitized at Different Magnifications*

Artery No.	Specified Magnification	Criteria							
		D1	D2	MT	LA	LC	IEL	MA	EEL
1	×20	1.01	1.01	0.33	1.03	1.02	1.01	0.97	0.99
	×40	1.04	1.03	0.38	1.08	1.04	1.04	1.01	1.03
6	×10	1.03	0.99	0.40	1.09	1.05	1.03	0.96	1.07
	×20	1.02	1.02	0.25	1.13	1.06	1.05	0.96	1.11
	×40	1.03	1.03	0.26	1.15	1.08	1.06	0.98	1.10
7	×10	0.94	1.00	0.33	0.98	0.99	1.00	1.01	0.98
	×20	0.96	0.98	0.24	1.00	1.01	1.03	1.02	0.99
	×40	0.97	1.01	0.26	1.03	1.02	1.05	1.03	1.00
8	×10	0.97	1.00	0.71	1.01	1.06	1.20	0.95	1.04
	×20	0.97	1.02	0.66	1.05	1.09	1.24	1.00	1.11
9	×10	0.99	1.01	0.51	0.99	0.99	1.11	0.98	1.17
	×20	1.00	1.01	0.53	1.00	1.01	1.22	1.02	1.23
10	×10	0.97	0.98	0.38	0.99	1.04	0.99	0.96	1.02
	×20	0.99	1.00	0.38	1.03	1.06	1.04	0.97	1.05
	×40	1.00	1.02	0.38	1.06	1.08	1.07	1.03	1.09
11	×10	0.99	0.99	0.86	0.97	1.04	1.05	0.99	1.01
	×20	1.01	1.01	0.87	1.01	1.04	1.06	1.02	1.06
13	×10	0.99	0.99	0.97	0.97	1.00	1.18	0.98	1.14
	×20	1.02	1.02	0.99	1.00	1.04	1.30	1.02	1.20
14	×10	1.01	0.97	0.49	1.01	0.99	1.06	0.87	1.16
	×20	1.01	0.96	0.24	1.05	1.01	1.14	0.81	1.23
	×40	1.00	0.96	0.25	1.05	1.01	1.19	0.77	1.24
15	×10	1.02	0.96	0.40	1.02	1.01	1.10	0.85	1.02
	×20	1.04	0.98	0.24	1.05	1.07	1.15	0.88	1.11
	×40	1.04	1.00	0.26	1.06	1.10	1.16	0.89	1.12

*Values are expressed as the ratio of measurement at the specified magnification to the measurement at the lowest magnification (×4 for all arteries except No. 1). D1 and D2 indicate right-angle external diameters; MT, medial thickness; LA, luminal area; LC, luminal circumference; IEL, internal elastic lamina; MA, medial area; and EEL, external elastic lamina.

Table 5.—Digitizable Muscular Pulmonary Arteries

Subject No.	Lung, No. of Sections	Screening, No. of Arteries			
		First	Second	Third	Common to All
1	Injected, 12	28	28	30	26
	Uninjected, 12	31	30	33	26
2	Injected, 5	38	38	37	32
	Uninjected, 11	19	17	17	16
3	Injected, 11	48	45	49	42
	Uninjected, 11	11	13	13	10
4	Injected, 9	14	15	15	14
	Uninjected, 10	27	27	25	22

COMMENT

This study has established a new technique for measuring the structural components of muscular pulmonary arteries in which artery size is expressed in terms of the length of the internal elastic lamina; we consider the technique to have advantages over previously used techniques.

The general advantages relate to the ease with which the measurements of arteries can be obtained from either histologic sections or from photographs, as tiresome calculations to account for magnification are unnecessary. Storage of the data on tape is a further asset as it enables the operator, by simple programming, to handle the data in a variety of ways.

The specific advantages of the technique may be discussed in the light of the two aims of the present study. We hoped first to establish that the medial area and length of internal elastic lamina are more reliable indicators of medial hypertrophy and vessel size than other, previously used measurements, and second, that the new method of measuring these two criteria is an improvement on other, currently used methods.

Repeatability of the measurements of all criteria of the muscular pulmonary arteries was excellent, with the notable exception of medial thickness. The very poor repeatability of this measurement in uninjected arteries adds further criticism to that already levelled[12] at studies that have used this measurement. It was surprising, however, to find that the repeatability was equally poor in injected arteries, although comments of this nature have been made.[17] Although diameter measurements were consistently repeatable, the expression of medial thickness as a percentage of vessel diameter does not reduce the inherent error and the conclusions drawn from such studies may well be suspect.

Furthermore, the extent to which vasconstriction can affect the percentage wall thickness measurement is particularly well illustrated by one of the 15 arteries that was included in the initial sections of the present study (Fig 3). The observed percentage wall thickness—[Mean (M1 + M2 + M3 + M4)]/[Mean (D1 + D2)] × 100%—of this artery was 39.8%, whereas its true percentage wall thickness (derived using the medial area and length of internal elastic lamina measurements) was only 14.4%. Such findings raise serious

Table 6.—Percent Medial Thickness Values for the Muscular Pulmonary Arteries of Subject 1

Lung	Artery Population	External Diameter Range, μm* <100	100-199	200-299	300-399	400-499	≥500
Injected	Digitizable	0/ . . .	8/6.0 ± 1.0	7/5.1 ± 1.7	6/4.3 ± 1.1	3/5.0 ± 1.7	8/4.3 ± 0.6
	Total	22/10.5 ± 4.5	124/6.4 ± 1.6	74/5.0 ± 1.4	37/4.7 ± 1.2	19/4.3 ± 1.2	25/4.3 ± 1.1
Uninjected	Digitizable	2/14.7 ± 1.9	16/10.6 ± 3.0	9/11.1 ± 2.2	3/12.1 ± 1.9	2/9.6 ± 0.6	2/13.1 ± 0.2
	Total	82/14.0 ± 4.5	154/11.2 ± 3.0	66/11.3 ± 3.3	21/11.5 ± 3.1	6/12.4 ± 3.1	12/13.8 ± 3.3

*Number of arteries/percent thickness (mean ± SD) is given.

doubts about the validity of the frequently used wall thickness methods in the assessment of medial hypertrophy.

Measurements of medial area, unlike medial thickness, were generally very repeatable and varied little with magnification. Where evident, poorer repeatability was linked to the structure, eg, the very thin media of injected arteries, or very small arteries. The former is of no real importance as the new technique is intended for use on uninjected material. With regard to very small arteries, repeatability was within 10%, which we considered to be acceptable in view of the difficulties that are involved in accurately measuring very small areas.

With regard to indicators of artery size, we consider that the problems associated with diameter are not satisfactorily overcome by distention of the arteries. First, there is the problem of deciding which distending pressure should be used—the pressure that is measured during life using cardiac catheterization techniques, or a standard hypertensive pressure. Regardless of which pressure is chosen, it is difficult to maintain injection of the pulmonary artery at that pressure. It has also been pointed out that the pulmonary arteries sometimes dilate excessively and unpredictably[35] and that changes in the intima may affect the distensibility of an artery.[34] A further criticism is our observation that some arteries appeared to have resisted distention and showed marked crenation of the elastic laminae. These problems and observations lead to reservations about the injection of pulmonary arteries and the use of diameter as an indicator of vessel size, even though it is a measurement that is consistently repeatable and varies little with magnification.

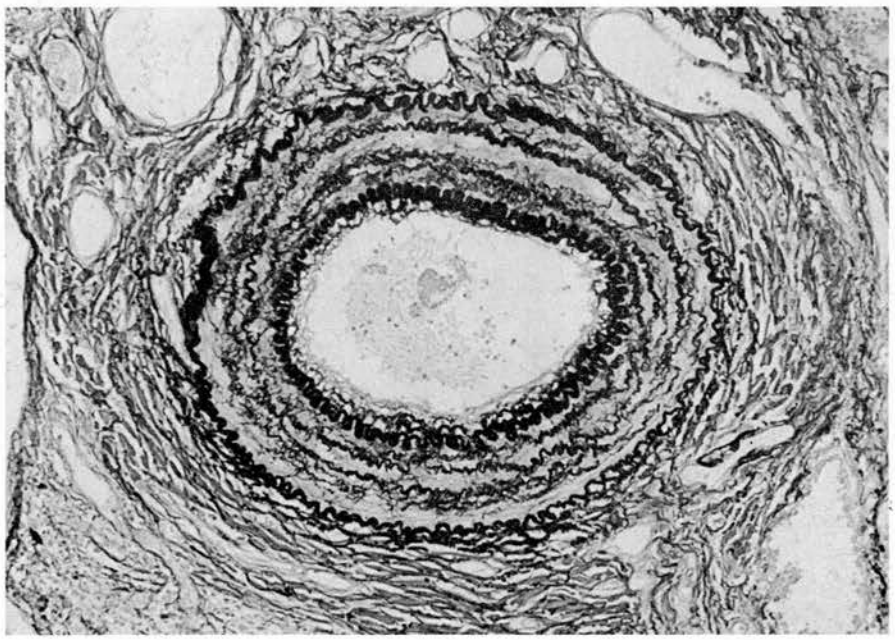

Fig 3.—Muscular pulmonary artery (No. 13) showing marked vasoconstriction (elastic van Gieson, ×230).

The definition of artery size by its position relative to the accompanying airway is also considered to be unsatisfactory because of the disparity between the branching patterns of the pulmonary artery and bronchial tree.[36]

The area of the arterial intimal nuclei that has been used by some workers[19] is a criterion that fulfills the requirement of being unaffected by vasoconstriction or dilation; however, its major disadvantage as an indicator of size is that the assessment of medial hypertrophy is limited to those arteries in which intimal change is absent. As changes in the intima of pulmonary arteries may be brought about by natural processes such as aging, this method of assessment will exclude not only any diseased arteries but also some otherwise normal arteries. The value of measurements that are obtained from such a highly selected group of arteries is questionable.

Relating the medial area to the area of histologic section,[14] although it is theoretically sound, gives an overall impression of the degree of medial hypertrophy that is present but does not provide data for the individual artery. With this method, sampling of the lung must be strictly controlled as the measurements will undoubtedly be affected by the site of the samples.

If data for the individual artery are to be obtained, then the most sensible indicator of artery size is that which is based on the length of the internal elastic lamina. This size indicator has several advantages over the others. It is unaffected by vasoconstriction and measurements are potentially possible on all of the arteries that satisfy certain criteria regarding the angle of the cut. Furthermore, the concept of theoretically "unwrinkling" a vessel to determine its uncollapsed/uncon-

stricted size is one which is readily understood.

Some researchers may consider the fact that only a small proportion of the muscular pulmonary artery population is digitizable to be a major drawback of our method of measurement. We have, however, shown that our criteria for digitizability are stringent and also that the digitizable arteries are representative of the total population as assessed by muscle thickness expressed as a percentage of the external diameter. It was somewhat unfortunate that this particular aspect of the study had to be based on a measurement that showed poor repeatability, but this was considered to be justifiable on the grounds that no other measurement of the media was possible on all of the arteries. Although the new method of measurement is intended for use on uninjected material, both injected and uninjected lungs were studied with respect to the selection of vessels that were considered digitizable and the comparison of digitizable vessels with the total population. We wished to establish that our comments with regard to these two factors were equally applicable to uninjected or injected arteries; the reasons for this relate to the second part of this study, in which the effects of arterial distention on measurements of the media and internal elastic lamina will be determined.

Several other researchers have defined artery size in terms of the length of the internal elastic lamina,[17,18,21-24,31,32] but their methods of measuring this criterion are time consuming and tedious. The most common methods involve tracing an image of the artery that is produced either by projection[18,21-24] or a microscope camera lucida,[17] and measuring the length of the internal elastic lamina on the tracing with a rotameter[17] or by attaching a thin cotton thread to the line of the lamina and measuring its length.[18,21-24] None of these studies mentioned the repeatability of the measurements that were obtained. Measurement of the length of the internal elastic lamina has also been obtained by stereologic counting methods.[37] Our technique is considered to have advantages over those other techniques simply because of the ease of obtaining the measurements and of data handling. The only problem that is associated with the measurement of the internal elastic lamina arises in extremely crinkled vessels that are viewed at low magnification. The group of arteries that is most affected are the larger vessels, but because measurements are just as easily obtained from photographs, the solution is simple.

In this report, we have limited ourselves to the discussion of measurements of the media and size of muscular pulmonary arteries. In fact other measurements are possible using the described technique; medial area measurements may be obtained for partially muscular arteries and measurements of the intima may be obtained for all classes of pulmonary artery. The latter measurements will be reported later.

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Program 2 may be obtained from the Institute of Occupational Medicine, 8 Roxburgh Pl, Edinburgh EH8 9SU, Scotland.

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